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(54) Title: PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND METHODS OF USE

(57) Abstract: The present invention provides isolated European-like porcine reproductive and respiratory syndrome viruses, polynucleotides, and polypeptides. The present invention also provides methods for making antibodies to the viruses and polypeptides, methods for detecting porcine reproductive and respiratory syndrome viruses, immunogenic compositions, and methods for treating a porcine subject at risk of infection by, or displaying symptoms of, a porcine reproductive and respiratory syndrome virus infection.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND METHODS OF USE

CONTINUING APPLICATION DATA

This application claims the benefit of the following U.S. Provisional Applications: Serial No. 60/181,041, filed February 8, 2000; Serial No. 60/193,220, filed March 30, 2000; Serial No. 60/206,624, filed May 24, 2000; Serial No. 60/215,373, filed June 29, 2000; and Serial No. 60/_____, filed January 5, 2001, docket number 110.0125 0164, entitled PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND METHOD OF DETECTION; each of which is incorporated by reference herein.

BACKGROUND

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the family *Arteriviridae* in the order *Nidovirales* (Cavanagh et al., *Virol.*, 176, 306–307 (1990)) that causes reproductive failure in breeding swine and respiratory problems in young pigs (see Rossow, *Vet. Pathol.*, 35, 1-20 (1998)). The syndrome was first recognized as a "mystery swine disease" in the United States in 1987 and was discovered in Europe in 1990. A strain of PRRSV that is prevalent in Europe has been isolated and is referred to as the Lelystad virus (Wensvoort et al., *Vet. Q.*, 13, 121-130 (1991)). A North American PRRSV, referred to as VR-2332, has been isolated (Collins et al., *J. Vet. Diagn. Investig.*, 4, 117-126 (1992)). The disease has also been referred to as Wabash syndrome, mystery pig disease, porcine reproductive and respiratory syndrome, swine plague, porcine epidemic abortion and respiratory syndrome, blue abortion disease, blue ear disease, abortus blau, and seuchenhafter spatabort der schweine. The disease is characterized by reproductive failure in pregnant sows and respiratory problems in pigs of all ages. The disease has a significant negative impact on the swine industry.

PRRSV is an enveloped positive single-stranded RNA virus. The 5'-capped and 3'-polyadenylated RNA of the virus is polycistronic, containing (5' to 3') two large replicase open reading frames (ORFs), 1a and 1b, and several smaller ORFs. In the infected cell, arteriviruses produce a nested set of six to eight major coterminal subgenomic mRNAs (sgmRNAs) each thought to express only the relative 5'-terminal ORF. These sgmRNAs have a leader sequence derived from the 5' end of the genome that is joined at specific leader-body junction sites located downstream by an unclear discontinuous transcription mechanism (Lai, *Adv. Exp. Med. Biol.*, 380, 463–471 (1995)). The sgmRNAs of PRRSV encode four glycoproteins (GP2 to 5, encoded by sgmRNAs 2 to 5), an unglycosylated membrane protein (M, encoded by sgmRNA 6), and a nucleocapsid protein (N, encoded by sgmRNA 7). The European prototype strain of PRRSV, Lelystad, contains all six of these proteins in the virus particle, but only the proteins encoded by ORFs 5 to 7 have conclusively been demonstrated to be in the virion of North American isolates.

Nucleotide and amino acid sequence comparisons of the 3'-terminal ORFs 2 to 7 have shown that there are significant differences between PRRSV strains native to Europe and those found in North America (Kapur et al., *J. Gen. Virol.*, 77, 1271–1276 (1996), Murtaugh et al., *Arch. Virol.*, 40, 1451-1460 (1995)). Substantial variation also occurs among North American PRRSV isolates. Genotypic comparison between strains VR-2332 and Lelystad has revealed that ORF 1a of VR-2332 is vastly different from that of Lelystad in both length and sequence, while ORF 1b is relatively conserved between the two strains of PRRSV. The 5' leader sequence of VR-2332 was 31 bases shorter than that of Lelystad and differed considerably in nucleotide sequence. Regional amino acid sequence comparisons also revealed that although the recognized functional domains of the ORF 1a proteins were present in both strains, the proteins were not well conserved between these domains. Thus, although these two PRRSV strains cause similar diseases, they are different in the genes encoding structural proteins.

PRRSV continues to cause significant economic losses throughout the world. Vaccines are available, but they are based on one PRRSV strain, and there is evidence that PRRSV strains vary at the antigenic and genetic levels. In addition, since the virus was identified in Europe and in the United States, new disease phenotypes have continued to emerge.

SUMMARY OF THE INVENTION

The present invention represents the identification of a novel porcine reproductive and respiratory syndrome virus (PRRSV). It is known to the art that there is a great deal of nucleotide sequence variation between European PRRSV associated with European outbreaks of mystery swine disease (MSD) and North American PRRSV associated with North American outbreaks of MSD. As used herein the terms "European PRRSV" and "European strain" are used interchangeably and refer to strains of PRRSV that are prevalent in Europe. An example of a "European PRRSV" that is known to the art is the prototypic European strain, Lelystad, which is available from the Collection Nationale De Cultures De Microorganisms, Institut Pasteur, France, as deposit number I-1102 (see Wensvoort et al., U.S. Patent 5,620,691). The nucleotide sequence of the Lelystad strain is available at Genbank Accession Number NC_002533. As used herein the terms "North American PRRSV" and "North American strain" are used interchangeably and refer to strains of the PRRSV that are prevalent in North America. An example of a "North American PRRSV" that is known to the art is the prototypic North American strain, VR-2332, which is available from the ATCC as deposit number VR-2332. The nucleotide sequence of the VR-2332 strain is available at Genbank Accession Number U87392.

The PRRSV described herein has not been described before, and was associated with a North American outbreak of MSD, but unexpectedly and surprisingly has a nucleotide sequence that has more similarity to European PRRSV strains, than to North American PRRSV strains. As used herein the phrase "European-like PRRSV" and "European-like strain" are used interchangeably and refer to PRRSV of the present invention. The characteristics of European-like PRRSV are described herein.

The present invention provides an isolated virus deposited under ATCC Accession Number _____, and an isolated cell comprising the virus. Also provided by the invention is an isolated virus that includes an RNA polynucleotide that includes the RNA nucleotide sequence corresponding to SEQ ID NO:1. The invention provides an isolated polynucleotide that includes the sequence SEQ ID NO:1. The isolated polynucleotide can have at least about 96 % identity with a polynucleotide having the sequence shown in SEQ ID NO:1 using a GAP algorithm with default parameters, wherein the polynucleotide replicates in a cell.

Also provided is a vector that includes a polynucleotide that includes the sequence shown in SEQ ID NO:1, and a polypeptide that includes an amino acid sequence selected from the group consisting of SEQ ID NO:2-10. The invention provides polypeptides that have an amino acid sequence having at least about 95 % identity to SEQ ID NO:2, at least about 99 % identity to SEQ ID NO:3, at least about 98 % identity to SEQ ID NO:4, at least about 94 % identity to SEQ ID NO:5, at least about 95 % identity to SEQ ID NO:6, at least about 91 % identity to SEQ ID NO:7, at least about 99 % identity to SEQ ID NO:9, or at least about 99.5 % identity to SEQ ID NO:10.

The invention provides an antibody that specifically binds a European-like porcine reproductive and respiratory syndrome virus (PRRSV), and a method of making an antibody. The method includes administering to an animal a virus particle that includes an RNA polynucleotide that includes the RNA nucleotide sequence corresponding to SEQ ID NO:1, or a polypeptide that includes an amino acid sequence selected from the group consisting of SEQ ID NO:2-10, or a polynucleotide encoding the polypeptide. The particle, polypeptide, or polynucleotide is administered in an amount effective to cause the production of an antibody specific for the virus particle. The antibody can be a polyclonal antibody or a monoclonal antibody, and the method can further include isolating the antibody. Also provided is the antibody produced by the method.

Methods for detecting a PRRSV are also provided. A method includes contacting a virus particle, for instance from a biological sample, with an antibody of the present invention under conditions to form a complex with a virus particle, and detecting the complex, wherein the presence of the complex indicates the presence of a PRRSV. The method can also be used to detect PRRSV in a porcine subject. Also provided is a kit for use in detecting PRRSV in a porcine subject. The kit includes the antibody of the invention and instructions for using the antibody.

Methods for detecting the presence of a European-like PRRSV are also provided. The methods include contacting a viral polynucleotide with a first primer and a second primer under conditions suitable to form a detectable amplification product. The first primer includes a nucleotide sequence that is complementary to nucleotides 2268 and 2269 of SEQ ID NO:1 or the complement thereof. The method further includes detecting an amplification product, wherein the detection indicates that the viral polynucleotide is a European-like PRRSV. Examples of first primers that can be used include 5'ATCGGGAATGCTCAGTCCCCTT (SEQ ID NO:12), and 5'-AAGGGGACTGAGCATTCCCG (SEQ ID NO:14). The method can also be used for detecting the presence of a European-like PRRSV in a porcine subject, and includes contacting a biological sample of a porcine subject with the first primer and the second primer. The biological sample preferably includes lung tissue.

Also provided by the invention is a kit for use in detecting PRRSV in a porcine subject. The kit includes the first primers and second primers of the invention suitable for use in amplification of a portion of a PRRSV and instructions for using the primer pair. Another kit provided by the invention is for use in detecting antibody to PRRSV in a porcine subject. The kit includes the virus of the invention and instructions for using the virus.

Further provided by the invention is an immunogenic composition. The composition includes an attenuated or inactivated PRRSV that includes a polynucleotide having at least about 96 % identity with a polynucleotide having the sequence shown in SEQ ID NO:1 using a GAP algorithm with default

parameters. The immunogenic composition may include a polypeptide selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, an immunogenic analog thereof, an immunogenic fragment thereof, or a combination thereof.

Methods of treating a porcine subject at risk of infection with a PRRSV or displaying symptoms of a PRRSV infection are also provided. The methods include administering to the animal an immunogenic composition that includes an attenuated or inactivated PRRSV that includes a polynucleotide having at least about 96 % identity with comprising an RNA polynucleotide comprising the RNA nucleotide sequence corresponding to SEQ ID NO:1 using a GAP algorithm with default parameters. The immunogenic composition is administered in an amount effective to cause an immune response to the PRRSV. The immunogenic composition can include a polypeptide selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, an immunogenic analog thereof, an immunogenic fragment thereof, or a combination thereof. Alternatively, the porcine subject can be administered a neutralizing antibody in an amount effective to treat the porcine subject.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. DNA nucleotide sequence of a portion of the positive strand of the genome of the European-like strain (SEQ ID NO:1). The RNA sequence that corresponds to SEQ ID NO:1 and is present in a viral particle has uracil (U) nucleotides instead of the thymidine (T) residues. Rows 1, 2, and 3 under the nucleotide sequence represent the three different reading frames. The predicted amino acid sequences encoded by the European-like strain are depicted for some

predicted open reading frames, including: SEQ ID NO:2 (ORF1a), SEQ ID NO:3 (ORF1b), SEQ ID NO:4 (ORF2), SEQ ID NO:5 (ORF3), SEQ ID NO:6 (OFR4), SEQ ID NO:7 (ORF5), SEQ ID NO:8, SEQ ID NO:9 (ORF6), and SEQ ID NO:10 (ORF7).

Figure 2. DNA nucleotide sequence of a portion of the positive strand of the genome of the European-like strain (nucleotides 1,830 to 2,618 of SEQ ID NO:1) compared to a portion of the DNA nucleotide sequence of the prototypic European strain Lelystad (SEQ ID NO 11, which corresponds to nucleotides 1,981 to 2,820 of Genbank Accession Number NC_002533). In SEQ ID NO:11, the upper case nucleotides signify aligned non-identical nucleotides; lower case nucleotides signify unaligned nucleotides; dashes signify aligned identical nucleotides; and dots signify a gap.

DETAILED DESCRIPTION OF THE INVENTION

Polynucleotides

The present invention is based on the the identification of a novel porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped positive single-stranded RNA virus. Accordingly, the present invention provides isolated polynucleotides. Preferably, an isolated polynucleotide can replicate in a cell. Preferably, an isolated polynucleotide of the present invention is no greater than about 15.3 kilobases. Whether an isolated polynucleotide can replicate in a cell can be determined by inserting the polynucleotide into an expression vector, producing an infectious RNA, introducing the infectious RNA to a cells, and evaluating if the infectious RNA causes the cell to produce virus particles. These methods are described in greater detail herein. A preferred example of a polynucleotide of the present invention is SEQ ID NO:1 (Figure 1). This polypeptide is a portion of a polynucleotide obtained from a European-like PRRSV. Preferably, the European-like PRRSV is one having the strain designation MND99-35186, and deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia, 20110-2209, USA, on July 7, 2000 (granted ATCC Accession Number _____), or deposited with

the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia, 20110-2209, USA, on February 8, 2001 (granted ATCC Accession Number _____). The deposits were made under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. It is expected that the complete nucleotide sequence of the PRRSV disclosed in SEQ ID NO:1 will include additional nucleotides at the 5' end of the polynucleotide. Specifically, it is expected that about 100 to about 200, preferably about 150, additional nucleotides can be present at the 5' end of SEQ ID NO:1 when the nucleotide sequence of the entire PRRSV represented by SEQ ID NO:1 is determined. It should be noted that while SEQ ID NO:1 is a DNA sequence, the present invention contemplates the corresponding RNA sequence, and RNA and DNA complements thereof, as well.

As used herein, an "isolated" substance is one that has been removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically synthesized. For instance, a polypeptide, polynucleotide, or virus particle of this invention can be isolated. Preferably, a polypeptide, polynucleotide, or virus particle of this invention is purified, i.e., essentially free from any other type of polypeptide, polynucleotide, or virus particle and associated cellular products or other impurities. As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded DNA and RNA. Unless otherwise noted, a polynucleotide includes the complement thereof. The nucleotide sequence of the complement of a polynucleotide can be easily determined by a person of skill in the art. A polynucleotide may include nucleotide sequences having different functions, including for instance coding sequences, and non-coding sequences such as regulatory sequences and/or non-translated regions. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. A polynucleotide can be linear or circular in topology. A polynucleotide can be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment.

"Polypeptide" as used herein refers to a polymer of amino acids and does

not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like.

The terms "coding region" and "coding sequence" are used interchangeably and refer to a polynucleotide region that encodes a polypeptide and, when placed under the control of appropriate regulatory sequences, expresses the encoded polypeptide. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. A regulatory sequence is a polynucleotide sequence that regulates expression of a coding region to which it is operably linked. Nonlimiting examples of regulatory sequences include promoters, transcription initiation sites, translation start sites, translation stop sites, and terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

"Complement" and "complementary" refer to the ability of two single stranded polynucleotides to base pair, i.e., hybridize, with each other, where an adenine of one polynucleotide will base pair to a thymine of a second polynucleotide and a cytosine of one polynucleotide will base pair to a guanine of a second polynucleotide. Two polynucleotides are complementary to each other when a nucleotide sequence in one polynucleotide can base pair with a nucleotide sequence in a second polynucleotide. For instance, 5'-ATGC and 5'-GCAT are complementary. The terms complement and complementary also encompass two polynucleotides where one polynucleotide contains at least one nucleotide that will not base pair to at least one nucleotide present on a second polynucleotide under the hybridization conditions described below. For instance the third nucleotide of each of the two polynucleotides 5'-ATTGC and 5'-GCTAT will not base pair, but these two polynucleotides are complementary as

defined herein.

The present invention also provides isolated polynucleotides that correspond to the coding regions present in SEQ ID NO:1. These coding regions are shown in Table 1.

Table 1. Coding regions of SEQ ID NO:1

Nucleotides of SEQ ID NO:1 corresponding to the coding region.	Polypeptide encoded by the coding region.	SEQ ID NO of the polypeptide.
71 to 7,210	ORF1a	SEQ ID NO:2
7,207 to 11,583	ORF1b	SEQ ID NO:3
11,594 to 12,343	ORF2	SEQ ID NO:4
12,202 to 12,994	ORF3	SEQ ID NO:5
12,744 to 13,295	ORF4	SEQ ID NO:6
13,292 to 13, 897	ORF5	SEQ ID NO:7
13,449 to 13,775	not applicable	SEQ ID NO:8
13,885 to 14,406	ORF6	SEQ ID NO:9
14,396 to 14,782	ORF7	SEQ ID NO:10

The present invention also includes polynucleotides having structural similarity to SEQ ID NO:1 or to a coding region present in SEQ ID NO:1. The similarity is referred to as "percent identity" and is determined by aligning the residues of the two polynucleotides (i.e., the nucleotide sequence of a candidate polynucleotide and the nucleotide sequence of SEQ ID NO:1 or a coding region of SEQ ID NO:1) to optimize the number of identical nucleotides along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of shared nucleotides, although the nucleotides in each sequence must nonetheless remain in their proper order. A candidate polynucleotide is the polynucleotide that has the nucleotide sequence being compared to SEQ ID NO:1 or to a coding region present in SEQ ID NO:1 (e.g., nucleotides 71 to 7,210 of SEQ ID NO:1). A candidate polynucleotide can be isolated from an animal, preferably a pig infected with PRRSV, or can be produced using recombinant techniques, or

chemically or enzymatically synthesized. Preferably, two nucleotide sequences are compared using the GAP program of the GCG Wisconsin Package (Genetics Computer Group, Madison, Wisconsin) version 10.0 (update January 1999). The GAP program uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.*, 48, 443-453 (1970)) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Preferably, the default values for all GAP search parameters are used, including scoring matrix = NewsgapDNA.cmp, gap weight = 50, length weight = 3, average match = 10, average mismatch = 0. In the comparison of two nucleotide sequences using the GAP search algorithm, structural similarity is referred to as "percent identity." Preferably, a polynucleotide includes a nucleotide sequence having a structural similarity with a coding region of SEQ ID NO:1 of at least about 96 %, more preferably at least about 98 %, most preferably at least about 99 % identity.

Another isolated polynucleotide provided by the invention is an RNA polynucleotide to which an oligonucleotide having the sequence AGAGCGGGAACAGAATCCTTCCCACCTTTAGCGGTACGCTTG (SEQ ID NO:18) hybridizes. Preferably, the RNA polynucleotide replicates in cells to form virus particles. Such an RNA is referred to as an infectious RNA. The production and testing of infectious RNAs are described in greater detail below. Preferably, hybridization conditions include denaturing about 1 μg total RNA with glyoxyl and electrophoresing through a 2% agarose gel, transferring to a nylon membrane (MagnaGraph, MSI, Westboro, MA), and crosslinking to the membrane by ultraviolet light. Preferably, the oligonucleotide is 3′-end radiolabeled, for instance with [α^{32}P]dATP (Amersham Life Science, Arlington Heights, IL) and terminal deoxynucleotide transferase (TdT) (Promega Corporation, Madison, WI). Preferably, hybridization conditions include incubation of the membrane containing the crosslinked RNA with the labeled oligonucleotide in a hybridization solution, for instance QuikHyb (Stratagene, La Jolla, CA) at 68°C for 16 hours. The membrane is then washed 3 times in a solution containing 0.9 M sodium chloride/0.09 M sodium citrate/pH 7.0 (6xSSC) and 0.5% sodium dodecyl sulfate (SDS) at 78°C, and then exposed to

autoradiography film (NEN Life Science Products, Boston, MA) or a phosphoimaging screen (Molecular Dynamics, Inc., Sunnyvale, CA). It is expected that under these conditions, the oligonucleotide will not hybridize to the European PRRSV Lelystad or to the North American PRRSV VR-2332.

Preferably, a polynucleotide of the present invention includes a deletion when compared to the nucleotide sequence of European strain Lelystad, which is available at Genbank Accession Number NC_002533. When the nucleotide sequence of SEQ ID NO:1 and Genbank Accession Number NC_002533 are compared, nucleotides 2,419 to 2,470 of Genbank Accession Number NC_002533 are not present in SEQ ID NO:1. Nucleotides 2268 and 2269 of SEQ ID NO:1 are immediately 5' (upstream) and 3' (downstream) of this deletion. Thus, those polynucleotides of the present invention that include nucleotides 2268 and 2269 of SEQ ID NO:1 include this deletion. The presence of this deletion is useful in distinguishing between a polynucleotide of the present invention and some PRRSV clinical isolates (described in greater detail herein).

The isolated polynucleotides of the present invention can be obtained from a virus particle. As used herein, the terms "virus particle" and "viral particle" are used interchangeably and refer to a PRRSV particle. A virus particle includes an RNA polynucleotide that will reproduce in a cell, for instance a cell in a pig and/or a cultured primary (i.e., freshly isolated) porcine alveolar macrophage, under the appropriate conditions. A virus particle also includes an envelope that surrounds the polynucleotide. A virus particle is typically obtained from a pig presenting symptoms of mystery swine disease (MSD), including abortion, anorexia, fever, lethargy, pneumonia, red/blue discoloration of ears, labored breathing (dyspnea), and increased respiratory rate (tachypnea). While not intending to be limiting, a virus particle can be obtained from such a pig by the removal of tissue, preferably lung tissue, followed by microscopic examination of the tissue for thickened alveolar septae caused by the presence of macrophages, degenerating cells, and debris in alveolar spaces. These characteristics indicate the presence of an infection by a PRRSV. The lung or other porcine tissue is then homogenized with a pharmaceutically

acceptable aqueous solution (such as physiological saline, Ringers solution, Hank's Balanced Salt Solution, Minimum Essential Medium, and the like) such that the tissue includes about 10 percent weight/volume amount of the homogenate. The virus can be isolated by low speed centrifugation as described in Example 1 to form a homogenate. Alternatively, the virus can be isolated by passing the homogenate through filters with pore diameters in the 0.05 to 10 micron range, preferably through a series of 0.45, 0.2 and 0.1 micron filters, to produce a homogenate containing the PRRSV. As a result, the homogenate contains viral particles having a size no greater than about 1.0 micron, preferably no greater than about 0.2 to 0.1 micron. Other tissues, including fetal tissue, may also be used to recover virus. Typically, such a virus particle is then grown *in vivo* (i.e., within the body of a subject) or in cell culture (i.e., *in vitro*) to produce more virus particles. This process of infecting an animal or a cell in culture, allowing the virus to reproduce, and then harvesting the newly produced virus is referred to herein as passaging the virus. Optionally, the virus is purified.

The homogenate described above can be passaged in cell culture by inoculation into a series of cultured cells. Cultured cells can be mammalian organ cells such as kidney, liver, heart and brain, lung, spleen, testicle, turbinate, white and red blood cells and lymph node cells, as well as insect and avian embryo preparations. Preferably, the cell is a primary porcine alveolar macrophage. Preferably, primary porcine alveolar macrophages are isolated from at least two pigs, and the primary porcine alveolar macrophages from each pig are not combined. It has been observed that there is some variability in the ability of the virus of the present invention to replicate in primary porcine alveolar macrophage, and the use of primary porcine alveolar macrophages from more than one pig significantly increases the ability to passage the virus in the macrophages. Culture media suitable for these cell preparations include those supporting mammalian cell growth such as serum (for instance, fetal calf serum or swine serum) and agar, blood infusion agar, brain-heart infusion glucose broth and agar and the like. After inoculating cultured cells with homogenate and growing the culture, individual clumps of cultured cells can be harvested

and reintroduced into sterile culture medium with cells. Alternatively and preferably, supernatants from cultured cells are subjected to low speed centrifugation and used to inoculate sterile culture medium containing cells.

Whether an isolated, preferably purified, virus particle obtained in this way is able to cause MSD can be determined by inoculation of 3 to 4 week old pigs as described in Example 1, or by the methods of Terpstra et al., (*Vet. Q.*, 13, 131-136 (1991)), and Collins et al., (U.S. Patent 5,846,805). These methods experimentally test if the viral particle reproduces late term abortion and reproductive failure in pregnant sows or clinical signs and microscopic lesions in gnotobiotic piglets similar to field outbreaks. Pigs experimentally inoculated in this manner can also be used for *in vivo* passage of the virus by collecting tissue and processing for the isolation of virus as described in Example 1.

After isolation, preferably purification, of the virus particle, the polynucleotide in the particle can be isolated by, for instance, treating the particle to remove the envelope. Methods for removing the envelope are known in the art and include, for instance, solubilizing with phenol:chloroform or guanidunium. Optionally, the polynucleotide is purified using methods known to the art, including, for instance, precipitating the polynucleotide.

The polynucleotides of the present invention can be present in a vector. A vector is a replicating polynucleotide, such as a plasmid, phage, cosmid, or artificial chromosome to which another polynucleotide (e.g., a polynucleotide of the present invention) may be attached so as to bring about the replication of the attached polynucleotide. When a polynucleotide of the present invention is in a vector the polynucleotide is DNA. When present in a vector, a polynucleotide of the invention can be referred to as a "recombinant polynucleotide." Construction of vectors containing a polynucleotide of the invention employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual.*, Cold Spring Harbor Laboratory Press (1989) or Ausubel, R.M., ed. *Current Protocols in Molecular Biology* (1994).

A vector can provide for further cloning (amplification of the polynucleotide), i.e., a cloning vector, or for expression of the polypeptide

encoded by a coding region present in the polynucleotide, i.e., an expression vector. Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable host cells for cloning or expressing the vectors herein are prokaryote or eukaryotic cells, and suitable vectors for cloning and/or expression in prokaryote and/or eukaryote cells are known to the art. Typically, when the vector is used to clone a polynucleotide, the host cell is a prokaryote. Suitable prokaryotes include eubacteria, such as gram-negative or gram-positive organisms. Preferably, *E. coli* is used. Host cells suitable for expression of the polypeptides of the invention are described in greater detail below.

The polynucleotide used to transform the host cell optionally includes one or more marker sequences, which typically encode a molecule that inactivates or otherwise detects or is detected by a compound in the growth medium. For example, the inclusion of a marker sequence can render the transformed cell resistant to an antibiotic, or it can confer compound-specific metabolism on the transformed cell. Examples of a marker sequence are sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, tetracycline, neomycin, and formulations of phleomycin D1 including, for example, the formulation available under the trade-name ZEOCIN (Invitrogen).

An expression vector optionally includes regulatory sequences operably linked to the coding sequence. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell. Examples of promoters for use in vectors present in prokaryotic cells include *lac*, *lac*UV5, *tac*, *trc*, T7, SP6 and *ara*.

Promoter sequences are known for eukaryotes. Most eukaryotic coding sequences have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is the

CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be a signal for addition of the poly A tail to the 3' end of the coding sequence. All these sequences are suitably inserted into eukaryotic expression vectors.

Transcription of a coding sequence encoding a polypeptide of the present invention in mammalian host cells can be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, and Hepatitis-B virus.

Transcription of a coding sequence encoding a polypeptide of the present invention by eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually having about 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation- and position-independent, having been found 5' and 3' to coding sequences, within an intron as well as within the coding sequence itself. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, alpha-fetoprotein, and insulin). Enhancers from eukaryotic cell viruses are also known and include the SV40 enhancer on the late side of the replication origin, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the coding sequence encoding a polypeptide of the present invention, but is preferably located at a site 5' of the promoter.

An expression vector can optionally include a ribosome binding site (a Shine Dalgarno site for prokaryotic systems or a Kozak site for eukaryotic systems) and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a codon for which there exists no corresponding aminoacetyl-tRNA, thus ending polypeptide synthesis. The polynucleotide used to transform the host cell can optionally further include a transcription termination sequence. The rrnB terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is an often

used terminator that is incorporated into bacterial expression systems. Transcription termination sequences in vectors for eukaryotic cells typically include a polyadenylation signal 3' of the coding sequence.

Suitable host cells for expression vector that includes a polynucleotide encoding a polypeptide of the invention can be derived from multicellular organisms. Such host cells are capable of processing and glycosylation activities. Vertebrate or invertebrate culture can be used. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda*, *Aedes aegypti*, *Aedes albopictus*, *Drosophila melanogaster*, *Trichoplusia ni*, and *Bombyx mori* are known to the art.

Vertebrate cells can also be used as hosts. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (CAS-7, ATCC CRL-1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen. Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO); CHO-K1 (ATCC CCL-61); CHO-D; mouse sertoli cells (TM4); monkey kidney cells (CV1, ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (WI 38, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL 51); TRI cells; MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2), MARC-145 (Kim et al., *Arch. Virol.*, 133, 477-483 (1993)), and MA-104 (ATCC CRL-2378).

An expression vector of the present invention can be used to determine if a polynucleotide of the present invention replicates in a cell. A polynucleotide of the invention replicates in a cell if the cell culture shows signs of cytopathic effect (CPE) as described in Example 2, and/or if virus particles can be isolated from the cells. The polynucleotide present in the expression vector can be transcribed *in vitro* (i.e., cell free) to produce RNA transcripts. The RNA transcripts can be introduced into cultured cells, and incubated under conditions suitable for replication of a PRRSV. RNA transcripts that replicate will use the

cellular machinery (including, for instance, ribosomes, and tRNAs) to replicate. The culture can be assayed as described in Example 2 for CPE. The presence of CPE indicates the virus is able to replicate in a cell. Optionally, the virus particles produced by the cells can be isolated. This type of expression vector is often referred to in the art as an infectious cDNA clone, and the RNA produced by the expression vector is referred to as an infectious RNA. Methods for cloning the European PRRSV Lelystad and inserting it into a vector are known to the art (Meulenberg et al., *J. Virol.*, 72, 380-387 (1998)), and it is expected that the polynucleotides of the present invention can be used in this way to produce infectious RNAs. Moreover, the method of Meulenberg et al. can be used to make viral particles. Accordingly, a person of skill in the art can provide a polynucleotide of the present invention, for instance SEQ ID NO:1, introduce the polynucleotide into an expression vector, and produce infectious RNAs that could be introduced to cells to result in the production of virus particles. The cells transfected with an infectious RNA can be, for instance, BHK-21 cells, CL-2621 cells, MA-104 cells, MARC-145 cells, or primary porcine alveolar macrophages, preferably primary porcine alveolar macrophages. Methods for efficiently transfecting cells include the use of calcium chloride, and commercially available products known under the trade names LIPOFECTIN and LIPOFECTAMINE. Methods for efficiently transfecting primary porcine alveolar macrophages are known to the art (Groot Bramel-Verheige et al., *Virol.*, 278, 380-389 (2000)).

Polypeptides

The present invention is also directed to polypeptides, preferably isolated polypeptides, encoded by polynucleotides of the present invention. Preferably, a polypeptide of the present invention has immunogenic activity. "Immunogenic activity" refers to an amino acid sequence which elicits an immunological response in a subject. An immunological response to a polypeptide is the development in a subject of a cellular and/or antibody-mediated immune response to the polypeptide. Usually, an immunological response includes but is not limited to one or more of the following effects: the production of antibodies,

B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells (see, for example, de Antonio et al., *Vet. Immunol. Immunopathol.*, 61, 265-277 (1998), and Kwang et al., *Res. Vet. Sci.*, 67, 199-201 (1999)) directed specifically to an epitope or epitopes of the polypeptide fragment. As used herein, an antibody that can "specifically bind" or is "specific for" a virus particle and/or a polypeptide is an antibody that interacts only with an epitope of the antigen that induced the synthesis of the antibody, or interacts with a structurally related epitope. An antigen that "specifically binds" a European-like PRRSV is an antibody that does not specifically bind a European PRRSV, preferably a European PRRSV having deposit number I-1102, or a North American PRRSV, preferably a North American PRRSV having deposit number VR-2332. As used herein, the term "complex" refers to the combination of an antibody and a virus particle and/or a polypeptide that results when an antibody specifically binds to a virus particle and/or a polypeptide.

Preferred examples of polypeptides of the invention are SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10 (see Table 1). The present invention further includes polypeptides having structural similarity with the polypeptides of the present invention. The structural similarity is referred to as percent identity and is generally determined by aligning the residues of the two amino acid sequences (i.e., a candidate amino acid sequence and the amino acid sequence of one of SEQ ID NOs:2-10) to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. A candidate amino acid sequence is the amino acid sequence being compared to an amino acid sequence present in a preferred polypeptide of the present invention. Preferably, two amino acid sequences are compared using the GAP program of the GCG Wisconsin Package (Genetics Computer Group, Madison, Wisconsin) version 10.0 (update January 1999). The GAP program uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.*, 48, 443-453 (1970) to find the alignment of two complete sequences

that maximizes the number of matches and minimizes the number of gaps. Preferably, the default values for all GAP search parameters are used, including scoring matrix = BLOSUM62.cmp, gap weight = 8, length weight = 2, average match = 2.912, and average mismatch = -2.003. In the comparison of two amino acid sequences using the GAP search algorithm, structural similarity is referred to as "percent identity." Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:2 of at least about 95 %, more preferably at least about 97 %, most preferably at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:3 of at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:4 of at least about 98 %, more preferably at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:5 of at least about 94 %, more preferably at least about 96 %, most preferably at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:6 of at least about 95 %, more preferably at least about 97 %, most preferably at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:7 of, in increasing order of preference, at least about 91 %, at least about 93 %, at least about 95 %, at least about 97 %, most preferably at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:9 of at least about 99 % identity. Preferably, a polypeptide includes an amino acid sequence having a structural similarity with SEQ ID NO:10 of at least about 99.5 % identity.

The present invention further includes polypeptide analogs and polypeptide fragments, preferably immunogenic polypeptide analogs and immunogenic polypeptide fragments. Preferably, a polypeptide fragment is at least about 8, more preferably at least about 12, most preferably at least about 20 amino acids in length. Immunogenic analogs of polypeptides of the present invention include polypeptides having amino acid substitutions that do not eliminate the ability of the polypeptide to elicit an immunological response.

Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, aspartate, and glutamate. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH_2.

Immunogenic analogs, as that term is used herein, also include modified polypeptides. Modifications of polypeptides of the invention include chemical and/or enzymatic derivatizations at one or more constituent amino acids, including side chain modifications, backbone modifications, and N- and C-terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like. Immunogenic fragments of a polypeptide include a portion of the polypeptide containing deletions or additions of one or more contiguous or noncontiguous amino acids such that the resulting polypeptide is immunogenic.

The polypeptides of the present invention can be obtained from, for instance, a biological sample from a porcine subject infected with a European-like PRRSV that encodes the polypeptide. Preferably, the European-like PRRSV is one that includes SEQ ID NO:1, more preferably it is the European-like PRRSV having ATCC number _____. The polypeptide can be obtained from cultured cells, preferably primary porcine alveolar macrophages, that have, for instance, been infected with a European-like PRRSV that encodes the polypeptide or contain a recombinant polynucleotide, preferably a

polynucleotide of the invention, that encodes a polypeptide of the invention. Alternatively, the polypeptide can be obtained from a prokaryotic cell or a eukaryotic cell that contains an expression vector that includes a polynucleotide encoding a polypeptide of the invention. The polypeptides of the present invention can also be obtained by chemical synthesis.

Viruses

The present invention includes isolated European-like virus particles. The European-like virus particles of the present invention include a polynucleotide having structural similarity to SEQ ID NO:1, preferably at least about 96 %, more preferably at least about 98 %, most preferably at least about 99 % identity to SEQ ID NO:1. A preferred example of a virus particle is one that includes SEQ ID NO:1. More preferably, the virus particle is the virus having ATCC Accession Number _____. A virus particle of the present invention include an envelope, and can, when added to a cultured cell, can replicate to result in the production of more viral particles.

As discussed above, a virus particle of the present invention can be obtained from a pig presenting symptoms of MSD. A virus particle can be grown by passage *in vivo* or in cell culture. Passage *in vivo* includes inoculating a pig, for instance as described in example 1. Passage in cell culture includes exposing cultured cells to the virus particle and incubating the cells under conditions suitable for the virus to reproduce and produce more virus particles. Preferably, the cultured cells are not an immortalized or transformed cell line (i.e., the cells are not able to divide indefinitely). Preferably, primary porcine alveolar macrophages are used for passage in cell culture. The use of primary porcine alveolar macrophages is described in Example 2. A virus particle can also be obtained from cells transfected with an infectious RNA as described herein.

A virus of the present invention can be inactivated, i.e., rendered incapable of reproducing *in vivo* and/or in cell culture. Methods of inactivation are known to the art and include, for instance, treatment of a virus of the invention with a standard chemical inactivating agent such as an aldehyde

reagent including formalin, acetaldehyde and the like; reactive acidic alcohols including cresol, phenol and the like; acids such as benzoic acid, benzene sulfonic acid and the like; lactones such as beta propiolactone and caprolactone; and activated lactams, carbodiimides and carbonyl diheteroaromatic compounds such as carbonyl diimidazole. Irradiation such as with ultraviolet and gamma irradiation can also be used to inactivate the virus.

Also included in the present invention are attenuated European-like PRRSV (i.e., viruses having reduced ability to cause the symptoms of MSD in pigs), and methods of making an attenuated European-like PRRSV. Methods of producing an attenuated virus are known to the art. Typically, a virus of the present invention is passaged, i.e., used to infect a cell in culture, allowed to reproduce, and then harvested. This process is repeated until the virulence of the virus in pigs is decreased. For instance, the virus can be passaged 10 times in cell culture, and then the virulence of the virus measured. If virulence has not decreased, the virus that was not injected into the animal is passaged an additional 10 times in cell culture. This process is repeated until virulence is decreased. In general, virulence is measured by inoculation of pigs with virus, and evaluating the presence of clinical symptoms and/or LD_{50} (see, for instance, Example 1, Halbur et al., *J. Vet. Diagn. Invest.*, 8, 11-20 (1996), and Halbur et al., *Vet. Pathol.*, 32, 200-204 (1995), and Park et al., *Am. J. Vet. Res.*, 57, 320-323 (1996)). Preferably, virulence is decreased so the attenuated virus does not cause the death of animals, and preferably does not cause clinical symptoms of the disease.

Typically, a cell culture useful for producing an attenuated virus of the present invention includes cells of non-porcine mammal origin. Examples of non-porcine mammal cell cultures include, for instance, the cell line MA-104 (ATCC CRL-2378), the cell line MARC-145 (Kim et al., *Arch. Virol.*, 133, 477-483 (1993)), and the cell line CL-2621 (Baustia et al., *J. Vet. Diagn. Invest.*, 5, 163-165 (1993)). Preferably, a mixed cell culture is used for producing an attenuated virus of the present invention. In a mixed cell culture there are at least two types of cells present. Preferably, a mixed cell culture includes an immortalized or transformed cell line and a primary cell culture. A mixed cell

culture is particularly useful when a virus reproduces slowly, or not at all, in an immortalized or transformed cell line. Preferred examples of an immortalized or transformed cell line for use in a mixed cell culture include, for example, the cell line MARC-145 (Kim et al., *Arch. Virol.*, 133, 477-483 (1993)), and the cell line MA-104 (ATCC CRL-2378). Preferably, primary cell cultures for use in a mixed cell culture are porcine in origin. A preferred example of a primary cell culture for use in a mixed cell culture is primary porcine alveolar macrophages.

Methods of use

The virus particles, polynucleotides, polypeptides, and immunogenic analogs and immunogenic fragments thereof of the present invention can be used to produce antibodies. Laboratory methods for producing, characterizing, and optionally isolating polyclonal and monoclonal antibodies are known in the art (see, for instance, Harlow E. et al. *Antibodies: A laboratory manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1988). For instance, a virus of the present invention can be administered to an animal, preferably a mammal, in an amount effective to cause the production of antibody specific for the administered virus. Polypeptides of the present invention and immunogenic analogs and immunogenic fragments thereof can also be administered to an animal, preferably a mammal, to produce antibodies. Optionally, a virus particle or a polypeptide is mixed with an adjuvant, for instance Freund's incomplete adjuvant, to stimulate the production of antibodies upon administration. Preferably, the antibody is a monoclonal antibody.

Preferably, an antibody produced using a virus of the present invention, or a polypeptide or immunogenic analog or immunogenic fragment thereof, is a neutralizing antibody. A neutralizing antibody is one that prevents a virus of the present invention from reproducing in cell culture, preferably in primary porcine macrophages.

Optionally and preferably, antibody produced using a virus particle of the present invention, a polypeptide or polynucleotide of the present invention, or immunogenic analogs and immunogenic fragments thereof do not specifically bind to the European PRRSV deposited as I-1102 with the Collection Nationale

De Cultures De Microorganisms, Institut Pasteur, France, or to the North American PRRSV deposited as VR-2332 with the ATCC. Whether an antibody of the present invention specifically binds to either or both of those viruses can be determined using methods known to the art.

The present invention provides methods for detecting a PRRSV, preferably a virus of the present invention. These methods are useful in, for instance, detecting a PRRSV in an animal, detecting a PRRSV in a cell culture, or diagnosing a disease caused by a PRRSV. Preferably, such diagnostic systems are in kit form. Kits are described in greater detail below. In some aspects of the invention, detecting a PRRSV includes detecting antibodies that specifically bind to a virus of the present invention, a polypeptide of the present invention, and/or an immunogenic analog or immunogenic fragment thereof. The method includes providing a biological sample, preferably a liquid homogenate of a tissue sample, from a porcine subject. The subject can be suspected of harboring the PRRSV, or can be a member of a herd that is being screened for the presence of the PRRSV. Antibody is added to the biological sample and incubated under conditions to form a complex with a PRRSV in the biological sample. Preferably the antibody is produced using a virus particle of the present invention, a polypeptide or polynucleotide of the present invention, or an immunogenic analog or immunogenic fragment thereof. Preferably, the antibody does not specifically bind a European PRRSV or a North American PRRSV. The complex is then detected, and the presence of the complex indicates the presence of a PRRSV in the biological sample. The detection of antibodies is known in the art and can include, for instance, immunofluorescence and peroxidase. Typical formats in which antibodies of the present invention can be used include, for instance, enzyme linked immunosorbent assay (ELISA); radioimmunoassay (RIA), immunofluorescent assay (IFA), and western immunoassay.

As used herein, a "biological sample" refers to a sample of tissue or fluid obtained from a subject, including but not limited to, for example, lung or respiratory tract. A "biological sample" also includes samples of cell culture constituents including but not limited to the cells and media resulting from the

growth of cells and tissues in culture medium. The cells can be infected with PRRSV or can contain a vector that includes a polypeptide of the present invention, and preferably includes a coding region encoding a polypeptide of the present invention.

Other methods for detecting a PRRSV, preferably a European-like PRRSV, include the amplification of a polynucleotide, preferably by the polymerase chain reaction (PCR). The polynucleotide can be one that is, for instance, present in a biological sample from a porcine subject that is suspected of harboring the PRRSV, or a member of a herd that is being screened for the presence of the PRRSV. The polynucleotide can be obtained from an isolated, preferably purified, virus particle. When the polynucleotide is obtained from a virus particle, the polynucleotide is converted from an RNA polynucleotide to a DNA polynucleotide by reverse transcription (see, for instance, Example 3). In some aspects of the present invention, the methods to detect a European-like PRRSV and distinguish it from a European PRRSV and a North American PRRSV exploit the presence of a deletion present in European-like PRRSV. This deletion is described above in the section labeled "Polynucleotides."

In one aspect of detecting a PRRSV by amplification of a polynucleotide, the invention is directed to detecting a virus of the present invention under conditions where European PRRSV and North American PRRSV are not detected. The method includes contacting a viral polynucleotide that is suspected of being a European-like PRRSV with a primer pair and incubating under conditions to form a detectable amplified polynucleotide. As used herein, a "primer pair" refers to two single stranded polynucleotides that can be used together to amplify a region of a polynucleotide, preferably by a polymerase chain reaction (PCR). The polynucleotide that results from amplifying a region of a polynucleotide is referred to as an "amplification product" or an "amplified polynucleotide." The phrase "under conditions suitable to form a detectable amplification product" refers to the reactions conditions that result in an amplification product. For instance, in the case of a PCR, the conditions suitable to form a detectable amplification product include the appropriate temperatures, ions, and enzyme.

One of the primers of the primer pair is complementary to a portion of the viral polynucleotide that corresponds to nucleotides 2268 and 2269 of SEQ ID NO:1, or the complement thereof. The use of such a primer results in the production of an amplified polynucleotide when there is a deletion. In contrast, the use of such a primer with a European PRRSV will not result in the production of an amplified polynucleotide because there are about 51 nucleotides present between the nucleotides that correspond to nucleotides 2268 and 2269 of SEQ ID NO:1, or the complement thereof. For instance, the use of such a primer pair will not result in an amplified polynucleotide when the viral polynucleotide is from the Lelystad PRRSV. An example of a primer pair that can be used in this method includes forward primer 5'ATCGGGAATGCTCAGTCCCCTT (SEQ ID NO:12), which corresponds to nucleotides 2,255 to 2,276 of SEQ ID NO:1 and reverse primer Euro2714 5'-GCGCATAAGACAGATCCA (SEQ ID NO:13), which is expected to result in an amplified polynucleotide of about 467 nucleotides. Another primer pair is reverse primer: 5'- AAGGGGACTGAGCATTCCCG (SEQ ID NO:14), which corresponds to the complement of nucleotides 2,257 to 2,276 of SEQ ID NO:1 , and forward primer Euro20/ 5'-CAGAAGGGTTCGAGGAAG (SEQ ID NO:15), which is expected to result in an amplified polynucleotide of about 170 nucleotides.

In another aspect of detecting a PRRSV by amplification of a polynucleotide, the invention is directed to detecting a virus of the present invention under conditions where both European-like PRRSV and European PRRSV are detected, and the molecular weights of the amplified polynucleotides vary. The primer pair used in this aspect produces an amplified polynucleotide that includes the region of the deletion, i.e., nucleotides 2268 and 2269 of SEQ ID NO:1, and the corresponding nucleotides of a European PRRSV. When both a European-like PRRSV and European PRRSV are amplified, and the resulting amplified polynucleotides are compared, the amplified polynucleotide that results from the European-like PRRSV will have a molecular weight that is about 51 nucleotides less than an amplified polynucleotide from a European PRRSV. Methods of determining the

approximate molecular weight of an amplified polynucleotide are known in the art, and include, for instance, resolving the polynucleotide on an acrylamide or agarose gel.

An example of a primer pair that can be used in this method includes forward primer Euro1671/ 5'-GCCTGTCCTAACGCCAAGTAC (SEQ ID NO:16) and reverse primer /Euro3165-rc: 5'-CATGTCCACCCTATCCCACAT (SEQ ID NO:17), which results in an amplified polynucleotide in a European-like PRRSV of about 1,494 nucleotides, and an amplified polynucleotide in a European PRRSV of about 1,544 nucleotides. Other primer pairs include forward primer Euro20/ 5'-CAGAAGGGTTCGAGGAAG (SEQ ID NO:15) and reverse primer /Euro3207 5'-GCTTGGAACTGCGAGG (SEQ ID NO:19) (expected size of amplified polynucleotide from a European-like PRRSV: about 910 nucleotides); forward primer Euro20/ 5'-CAGAAGGGTTCGAGGAAG (SEQ ID NO:15) and reverse primer /Euro2714 5'-GCGCATAAGACAGATCCA (SEQ ID NO:13) (expected size of amplified polynucleotide from a European-like PRRSV: about 616 nucleotides). When these primers are used to amplify a European PRRSV, the size of the amplified polynucleotide is expected to be about 51 nucleotides greater.

In another aspect of detecting a PRRSV by amplification of a polynucleotide, the invention is directed to detecting a virus of the present invention under conditions where European PRRSV is detected and European-like PRRSV are not detected. In this aspect of the invention, at least one of the primers of a primer pair is complementary a portion of nucleotides 2,419 to 2,470 of the prototype European PRRSV, Lelystad (Genbank Accession number NC_002533), of the complement thereof. An example of a primer pair that can be used in this method includes forward primer Euro1/: 5'-TGAAGGTGCTCTGGTCT (SEQ ID NO:20) and reverse primer /Euro2: 5'-AAATTCCCGCCTACC (SEQ ID NO:21), which results in an amplified polynucleotide from a European PRRSV of about 51 nucleotides.

The present invention also provides a kit for detecting a virus of the present invention, and a kit for detecting a polypeptide of the present invention or an immunogenic analog or immunogenic fragment thereof. The kit includes

an antibody that specifically binds a virus of the present invention, a polypeptide of the present invention or immunogenic analog or immunogenic fragment thereof (when detecting the presence of the virus) or a primer pair as described herein (when amplifying a polynucleotide) in a suitable packaging material in an amount sufficient for at least one assay. Preferably, the antibody does not specifically bind to the European PRRSV deposited as I-1102 with the Collection Nationale De Cultures De Microorganisms, Institut Pasteur, France, or to the North American PRRSV deposited as VR-2332 with the ATCC. The present invention also provides a kit for detecting antibody to a virus of the present invention, a polypeptide of the present invention or an immunogenic analog or immunogenic fragment thereof. When detecting antibody to the virus, polypeptide, or immunogenic analog or immunogenic fragment thereof, the kit includes a virus of the present invention, a polypeptide of the present invention or immunogenic analog or immunogenic fragment thereof. Optionally, other reagents such as buffers and solutions needed to practice the invention are also included. Instructions for use of the packaged virus or polypeptide or primer pair are also typically included.

As used herein, the term "packaging material" refers to one or more physical structures used to house the contents of the kit. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. The packaging material has a label which indicates that the polypeptide or primer pair can be used for detecting a virus of the present invention. In addition, the packaging material contains instructions indicating how the materials within the kit are employed to detect a virus of the present invention. As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits a virus or a primer pair. Thus, for example, a package can be a glass vial used to contain milligram quantities of a primer pair, or it can be a microtiter plate well to which microgram quantities of a virus have been affixed.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter, such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions, and the like.

The present invention is also directed to vaccines and methods of treatment. Treatment can be prophylactic or, alternatively, can be initiated after the development of MSD in a porcine subject. A vaccine can include, for instance, an immunogenic composition or a neutralizing antibody. The term "vaccine" refers to a composition that, upon administration to a subject, will provide protection against a virus of the present invention. When the vaccine includes an immunogenic composition, administration to the subject will also produce an immunological response to a polypeptide and result in immunity. An immunogenic composition of the present invention can include an attenuated or inactivated virus of the present invention, and/or one or more polypeptides of the present invention or immunogenic analogs or immunogenic fragments thereof, and/or a polynucleotide.

As used herein, the term "immunogenic composition" refers to a composition or preparation administered in an amount effective to result in some therapeutic benefit or effect so as to result in an immune response that inhibits or prevents MSD in a subject, or so as to result in the production of antibodies to a PRRSV of the present invention. Both local and systemic administration is contemplated. Systemic administration is preferred.

A polynucleotide used in a vaccine of the invention is preferably one that includes a nucleotide sequence encoding a polypeptide on the present invention, or an immunogenic analog or immunogenic fragment thereof. The polynucleotide can include DNA, RNA, or a combination thereof. The polynucleotide can be supplied as part of a vector or as a "naked" polynucleotide. General methods for construction, production and administration of polynucleotide vaccines are known in the art, e.g. F. Vogel et al., *Clin. Microbiol. Rev.* 8:406-410 (1995); WO 93/02556; Felgner *et al.*, U.S. Patent No. 5,580, 859, Pardoll *et al.*, *Immunity* 3:165 (1995); Stevenson *et al.*, *Immunol. Rev.* 145:211 (1995); Molling, *J. Mol. Med.* 75:242 (1997); Donnelly

et al., *Ann. N.Y. Acad. Sci.* 772:40 (1995); Yang *et al.*, *Mol. Med. Today* 2:476 (1996); and Abdallah *et al.*, *Biol. Cell* 85:1 (1995)). A nucleic acid molecule can be generated by means standard in the art, such as by recombinant techniques, or by enzymatic or chemical synthesis.

Preferably, administration of a polynucleotide that is part of a vaccine includes the introduction of an expression vector that includes the polynucleotide. There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccine plasmids, including, for instance, the plasmid pVAX1 as the vector (InVitrogen Corporation, Carlsbad, CA). In addition, the vector construct can contain immunostimulatory sequences that stimulate the animal's immune system. Examples of immunostimulatory sequences include, for instance, sequences with CpG motifs, two 5' purines, an unmethylated CpG dinucleotide, or two 3' pyrimidines (see, for instance, Lowie et al., DNA Vaccines Methods and Protocols, Humana Press, Totowa, NJ (2000)). Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences encoding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF) or interleukin-12 (IL-12). The cytokines can be used in various combinations to fine-tune the response of the animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to produce an immune response. Alternatively, the vaccine vector can be a viral vector, including an adenovirus vector, and adenovirus associated vector, or a retroviral vector.

Immunogenic carriers can be used to enhance the immunogenicity of a vaccine that includes an immunogenic composition. Such carriers include but are not limited to other polypeptides, polysaccharides, liposomes, and bacterial cells and membranes. Polypeptide carriers may be joined to the attenuated or inactivated virus of the present invention, and/or a polypeptide of the present invention or an immunogenic analog of immunogenic fragment thereof to form fusion polypeptides by recombinant or synthetic means or by chemical coupling. Useful carriers and means of coupling such carriers to polypeptide antigens are known in the art.

The vaccine preferably includes a pharmaceutical carrier that is compatible with a porcine subject. The vaccine may be delivered orally, parenterally, intranasally or intravenously. Factors bearing on the vaccine dosage include, for example, the age, weight, and level of maternal antibody of the infected pig. The vaccine doses should be applied over about 14 to 28 days to ensure that the pig has developed an immunity to the MSD infection.

The vaccine of the present invention can be administered in a variety of different dosage forms. An aqueous medium containing the vaccine may be desiccated and combined with pharmaceutically acceptable inert excipients and buffering agents such as lactose, starch, calcium carbonate, sodium citrate formed into tablets, capsules and the like. These combinations may also be formed into a powder or suspended in an aqueous solution such that these powders and/or solutions can be added to animal feed or to the animals' drinking water. These compositions can be suitably sweetened or flavored by various known agents to promote the uptake of the vaccine orally by the pig.

For purposes of parenteral administration, the composition can be combined with pharmaceutically acceptable carrier(s) well known in the art such as saline solution, water, propylene glycol, etc. In this form, the vaccine can be parenterally, intranasally, and orally applied by well-known methods known in the art of veterinary medicine. The vaccine can also be administered intravenously by syringe. In this form, the vaccine is combined with pharmaceutically acceptable aqueous carrier(s) such as a saline solution. The parenteral and intravenous formulations of the composition may also include emulsifying and/or suspending agents as well, together with pharmaceutically acceptable diluent to control the delivery and the dose amount of the composition.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Detection of virus in infected pigs.

This example describes the isolation of a new PRRSV from infected pigs. The PRRSV was given the designation MND99-35186 and is referred to in these examples as European-like.

Methods

Three live, 3 to 4-day-old pigs from a herd with a clinical history of late-gestation abortions and weak-born pigs were submitted to the Minnesota Veterinary Diagnostic Laboratory (St. Paul, Minnesota). The pigs were euthanized by intravenous over dose of barbiturate and necropsied (autopsied).

Portions of lung, lymph nodes, brain, spleen, kidney, tonsil and heart were collected and pooled as one sample. The sample was treated for the isolation of porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), and swine influenza virus (SIV).

For isolation of PRRSV, samples were inoculated on MARC-145 cells and primary porcine alveolar macrophages. Before inoculation, the samples were treated as described by Rossow et al. (*Vet. Pathol.*, 32, 361-373 (1995)). Briefly, Hank's Balanced Salt Solution was added to the tissue sample or sera to make an approximately 10 % (vol/vol) suspension, and then homogenized. The homogenate was centrifuged a 4,133 x g for 20 minutes, and the supernatant removed and saved. The pelleted material was discarded. The conditions for inoculating MARC-145 cells and primary porcine alveolar macrophages are described below. In addition, serum from each pig was pooled together into one sample and then used to inoculate MARC-145 cells and primary porcine alveolar macrophages.

Portions of lung, lymph nodes, stomach, brain, liver, kidney, tonsil, heart and ileum (a section of the small intestine) were preserved in 10% formalin buffered with a mixture of dibasic sodium phosphate and monobasic sodium phosphate to yield pH 7.0. The tissues were incubated in the formalin for at

least 12 hours before subsequent use in assays. Portions of each tissue were paraffin embedded.

Formalin fixed tissues were stained by hematoxylin and eosin (H and E) staining.

Formalin fixed tissues were also assayed for PRRSV testing by immunohistochemical technique by the method of Christopher-Hennings et al., (*Vet. Pathol.*, 35, 260-267 (1998)). Briefly, formalin fixed, paraffin embedded lung was sectioned at approximately 4 microns and sections were applied to glass slides. Tissue sections were covered with the monoclonal antibody SDOW-17 and incubated in a humidified chamber. SDOW-17 is an anti-PRRSV monoclonal antibody that recognizes an epitope present on the Lelystad PRRSV and on the VR-23332 PRRSV. Antibody binding to PRRSV in the lung was identified using a modified avidin biotin complex method (Hsu et al., *J. Histochem. Cytochem.*, 29, 577-580 (1981)).

Lung sections were rapidly frozen in isopentane at -30°C. The frozen sections were examined for PRRSV by direct fluorescent antibody technique using the monoclonal antibody SDOW-17. Direct FA examination of tissue was done by the method of Rossow et al. (*Vet. Pathol.*, 32, 361-373 (1995)). Briefly, tissues were frozen, sectioned at approximately 5 microns and transferred to glass slides. Tissue sections were covered with fluoroscene-conjugated SDOW-17, an anti-PRRSV monoclonal antibody (obtained from E. Nelson, South Dakota State University, South Dakota). The tissue sections were then incubated in a humidified chamber for about 1 hour, and excess unbound antibody removed by washing in phosphate buffered saline. The presence of antibody binding to PRRSV in the lung was visualized with a fluorescent microscope.

Brain, lung, and liver samples were cultured on blood agar plates to detect the presence of aerobic bacteria..

Pooled tissues were also examined for leptospira using the method of Smith et al. (*Cornell Vet.*, 57, 517-526 (1967)).

Results

PRRSV was identified in lung sections from each pig by immunohistochemical and direct fluorescent antibody.

Light microscopic tissue lesions (H and E slides) were compatible with PRRSV infection. Histopathology of the lungs showed diffuse septal thickening by macropahges. Some alveoli contained necrotic cell debris. Lymph nodes were characterized by germinal centers filled with blast-lymphocytes and small foci of necrosis. The muscular layer in one stomach was characterized by lymphoplasmacytic perineuritis and perivasculitis. Brain, liver, kidney, tonsil, heart, and ileum did not have lesions.

Tests for PRV and SIV were negative and no bacterial pathogens were identified in tissues from the infected pigs.

PRRSV was isolated from pooled tissue homogenate and pooled sera cultured in the alveolar macrophages. However, few cells were infected with PRRSV. No PRRSV was isolated from either sample cultured in MARC-145 cells.

Because this PRRSV grew poorly in the alveolar macrophages, 3 to 4-week-old pigs from a documented PRRSV-free farm were inoculated (intramuscularly and intranasally) with approximately 1 ml of the virus obtained from the supernatant from the infected alveolar macrophages. The virus was diluted by adding about 9 mls of Hanks Balanced Salt Solution to about 1 ml of virus-containing supernatant. Clinically, the experimentally infected pigs exhibited the same symptoms of mild, transient signs of lethargy within about 1-2 days that are also seen after infection of a pig with Lelystad virus or VR-2332. Each infected pig seroconverted to the PRRSV infection. Seroconversion was measured by the IDEXX Elisa test (HerdChek-PRRSV, IDEXX Laboratories Inc. Westbrook, ME). Seroconversion was also measured by indirect fluorescent antibody test using Lelystad infected cells and the method of Yoon et al. (*J. Vet. Diagn. Invest.*, 4, 144-147 (1992)). The European-like PRRSV was re-isolated from infected pig tissues and serum, cultured in porcine alveolar macrophages, and identified in tissues by immunohistochemistry.

Example 2

Infection of porcine alveolar macrophages with PRRSV

Porcine alveolar macrophages were isolated by collection from PRRSV-negative pigs less than 6-weeks-old. Pigs were euthanized, and trachea and lungs removed and airways lavaged with sterile phosphate buffered saline. The phosphate buffered saline was made by combining 8.5 grams NaCl, 1.1 grams disodium phosphate, and 0.32 gram sodium monophosphate in 10 liters distilled water. The Porcine alveolar macrophages were concentrated by centrifugation, confirmed negative for PRRSV by isolation and examination using direct fluorescent antibody as described in Example 1, and used immediately or stored in liquid nitrogen at a concentration of 10^6 cells/ml. Frozen alveolar macrophages chould be used within 6 months.

Freshly harvested porcine alveolar macrophages (about 10^7) or frozen cells (10^6 cells) were plated on a 1 x 48 well plate or a 1 x 75 cm flask, and allowed to adhere for 4 hours to overnight in about10 to 25 ml RPMI-1640 complete medium. The cells cannot be allowed to incubate for more than one day before virus is added. RPMI-1640 complete medium is made by combining 500 ml RPMI-1640 medium containing 300 mg/liter L-glutamine, 25 mM HEPES [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] (Catalog # 10-041-CV, Mediatech Inc., Herndon, Virginia), 40 ml heat-inactivated Fetal Bovine Serum (Cat #12133-78P; JRH Bioscience, Inc., Lenexa, Kansas), 1 gram neomycin sulfate (Gibco Life Technologies, Rockville, Maryland), 40 ml Hanks Balanced Salt Solution (HBSS) (Gibco Life Technologies, #14180053, Rockville, Maryland) containing neomycin sulfate at a final concentration of 150 µg/ml medium), 66 ml HBSS containing penicillin G potassium salt (Sigma #P7794, St. Louis, Missouri), streptomycin sulfate (Sigma #S9137), and amphotericin B solubilized (Sigma A-9528) at a final concentration of 2500 U penicillin G, 0.45 mg streptomycin sulfate/ml medium, and 120 µg/ml amphotericin B/ml of medium.

The medium was removed, and the cells were washed in once ins HBSS, and the cells were infected with 1 ml of virus in 1 ml RPMI incomplete medium

(same as RPMI complete but without FBS added). The virus titer can vary, and when the virus is from tissue isolated from a PRRSV-infected pig, is unknown. HBSS was 500 mls HBSS supplemented with 5 mls of 100x neomycin (10 mg/ml) and 5 mls of 100x pennecillin (10,000 U ml), streptomycin (10 mg/ml), and fungizone (25 μg/ml).

The plate was gently rotated for 1 hour at room temperature. Nine mls of RPMI complete medium were added and the infected cells were incubated at 37°C. The cell were observed daily until 60-80% cytopathic effect (CPE) was seen (2-3 days). CPE appears as fragmented, vacuolated, malformed, shrunken cells.

Virus was isolated by removing the medium and centrifuging at about 4,000 x g for 10 minutes to remove cellular debris, and leave the virus in the supernatant.

The presence of PRRSV in the primary porcine alveolar macrophages was confirmed by staining with the SDOW-17 monoclonal antibody as described by Nelson et al. (*J. Clin. Microbiol.*, 34, 3184-3189 (1993)).

Example 3

Sequence analysis of virus

1. PRRSV RNA extraction:

Viral RNA was extracted from macrophage culture supernatants using QIAamp Viral RNA Mini Spin Kit (QIAgen, Inc., Valencia, California). Briefly, 280 μl cell culture fluid was added to 1120 μl Buffer AVL/Carrier RNA, pulse vortexed for 15 seconds, incubated at room temperature for 10 minutes, and centrifuged briefly. After this step, 1120 μl 100% ethanol was added to the reaction, pulse vortexed for 15 seconds, and centrifuged briefly. The reaction was then applied to a QIAamp spin column (in a 2 ml collection tube) in 630 μl aliquots (4X) and centrifuged at 6000 x g (800 rpm) for 1 minute, discarding flow- through each time. When all sample was applied to the filter, the QIAamp spin column was placed into a clean 2 ml collection tube, and again centrifuged. Buffer AW1 (500 μl) was added to the filter containing viral RNA, and this

reaction was centrifuged at 6000 x g (8000 rpm) for 1 minute. Buffer AW2 (500 µl) was added to the spin column and the column was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The collection tube was discarded, and the QIAamp spin column was place in a clean 1.5 ml centrifigue tube. Buffer AVE (60 µl) was added to the spin column, the column incubated at room temperature for 1 minutes, and then centrifuged at 6000 x g (8000 rpm) for 1 minutes.

2. RT-PCR: Two methods were used to reverse transcribe the viral RNA and obtain DNA for use in DNA sequence analysis. In general, the primers were selected to hybridize to an appropriate portion of SEQ ID NO:1 and amplify a DNA fragment that was then used in DNA sequencing reactions.

a. Qiagen OneStep RT-PCR:

Five microliters of extracted RNA were added to a reaction mix containing 1X Qiagen RT-PCR Buffer; 400 nM of each dATP, dCTP, dGTP and dTTP; 0.08 units / reaction RNase inhibitor (20 U/ µl, Perkin Elmer, Boston Massachusetts); 1/25 volume of Qiagen Enzyme mix; 300 nM each forward and reverse primer; to make a total reaction volume of 25 ml. The thermocycling conditions consisted of: 1 cycle 50°C for 30 minutes; 1 cycle of 95°C for 15 minutes; 35 cycles of 57°C for 30 seconds, 72°C for 45 seconds, 94°C for 45 seconds; 1 cycle of 57°C for 30 seconds; 1 cycle of 72°C for 10 minutes and a 4°C hold.

b. RT and PCR - 2 step reaction

b1. Reverse Transcription: Random primed cDNA was generated in the following way: 2 µl of 50 µM random hexamers were added to 6 µl of RNA extract. This was heated to 70°C for 5 minutes and quickly chilled on ice. Then 32 µl of a master mix containing 5 mM $MgCl_2$ (Perkin Elmer), 1X Perkin Elmer Buffer II (50 mM KCl, 10 mM Tris-HCl, (pH 8.3 at room temperature)), 1 mM dNTPs (Perkin Elmer), 1 U/µl RNase Inhibitor (20 U/ µl, Perkin Elmer), and 25 U/µl MuLV RT (murine leukemia virus reverse transcriptase; Perkin Elmer).

The thermocyling conmditions consisted of: 1 cycle of 22°C for 10 minutes; 1 cycle of 42°C for 15 minutes; 1 cycle of 95°C for 10 minutes, 1 cycle of 5°C for 5 minutes and hold at 4°C.

b2. PCR: Reactions tube containing 40 µl of 5 mM $MgCl_2$ (Perkin Elmer), 1X Perkin Elmer Buffer II, 300 nM forward primer, 300 nM reverse primer, and 0.25 U/µl Amplitaq Polymerase (Perkin Elmer) was added to 10 µl cDNA obtained from the reverse transcription (paragraph b1, above). Alternatively, to amplify longer section of random primed cDNA, Expand Long Template PCR Kit (Boehringer Mannheim, Indianapolis, Indiana) was used. The thermocycling conditions consisted of: 1 cycle of 93°C for 4 minutes; 35 cycles of 57°C for 30 seconds, 72°C for 45 seconds, 93°C for 45 seconds; 1 cycle of 57°C for 30 seconds; 1 cycle of 72°C for 10 minutes and a 4°C hold. (Annealing temperature would vary according to the primer pair utilyzed to amplify cDNA).

3. The results of each PCR were evaluated and prepared for DNA sequencing. Sequence analysis was performed by the Advanced Genetic Analysis Center (AGAC) (University of Minnesota, Minneapolis, Minnesota). using an ABI Model 377 DNA Sequencer.

I. Evaluation of PCR reactions on an agarose gel.

One gram of agarose was added to 100 ml of 1X TAE buffer. This was microwaved for 2 minutes, and 4 µl of 10 mg/ml EtBr was added to every 100 ml agarose. The gel was cast and allowed to solidify for about 15-30 minutes. Four µl of PCR product were mixed with 1 µl loading dye and added to the gel, which was run at 140 volts for 1 hour or 75 volts for 2 hours.

II. Purification of PCR product with Qiagen Qiaquick PCR purification Kit

For each sample to be purified, a column was placed into a collection tube. One hundred µl PB buffer were added to the 20 µl PCR reaction left in PCR tube, and mixed thoroughly. All of the PCR product/PB buffer mix was added to the column, and the column was spun for 1 minute at full speed in an

Eppendorf microfuge. The flow-through from collection tube was discarded, and the column was placed back in the tube. Seven hundred and fifty µl of PE buffer was added, and the column spun for another minute at full speed. After discarding the flow-through from collection tube, the column was spun for another minute at full speed to remove any residual PE buffer from the column. The column was transferred into a clean, microfuge tube, and 30 µl H_2O was added to the column and incubated for at least a minute at room temp. The column was spun for one minute at full speed. The PCR product/H_2O eluate in the microfuge tube and was ready to be added to the sequencing reaction.

Example 4

Detection of European-like PRRSV.

In this example, viral DNAs were amplified using primers that amplify European-like PRRSV, European PRRSV, and North American PRRSV. The amplified region included the deletion that is present in European-like PRRSV.

The viral RNA of Lelystad was obtained from supernatants of infected MA-104 cells, the viral RNA of VR-2332 was obtained from supernatants of infected MA-104 cells, and the viral RNA from European-like PRRSV was obtained from supernatants of infected primary porcine alveolar macrophages. cDNA of viral RNA was prepared as described above in Example 3.

The viral cDNAs were amplified using the primers Euro1671/: 5'-GCCTGTCCTAACGCCAAGTAC (SEQ ID NO:16) and /Euro3165-rc: 5'-CATGTCCACCCTATCCCACAT (SEQ ID NO:17). The amplification conditions are listed in Table 2.

Table 2. General PCR Conditions (for 50 uL reaction)

Component	Stock Concentration	Final Conc
$MgCl_2$	25 mM	5mM
Buffer II[1]	10X	1x
Forward Primer	15 uM	0.3 uM
Reverse Primer	15 uM	0.3 uM
Taq Polymerase	5 U/ul	0.25 U/ul

[1]Buffer II, manufactured by Perkin Elmer.

Results

Amplification of viral DNA from Lelystad, VR-2332, and European-like resulted in amplification products that migrated at the predicted molecular weights. As expected, the product of amplifying the European-like DNA migrated at about 1.5 kilobases, approximately 51 base pairs less than Lelystad.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

Sequence Listing Free Text

SEQ ID NO:1	Portion of nucleotide sequence of a porcine reproductive and respiratory syndrome virus
SEQ ID NOs:2-10	Polypeptides predicted from open reading frames of SEQ ID NO:1.
SEQ ID NO:11	Nucleotides 1,981 to 2,820 of the Lelystad virus

SEQ ID NOs:12-17	Primer
SEQ ID NO:18	Oligonucleotide
SEQ ID NOs:19-21	Primer

5

What is claimed is:

1. An isolated virus deposited under ATCC Accession Number _____.

2. An isolated cell comprising a virus deposited under ATCC Accession Number _____.

3. An isolated virus comprising an RNA polynucleotide comprising the RNA nucleotide sequence corresponding to SEQ ID NO:1.

4. An isolated polynucleotide comprising the sequence of SEQ ID NO:1.

5. An isolated polynucleotide wherein the the sequence is SEQ ID NO:1.

6. An isolated polynucleotide having at least about 96 % identity with a polynucleotide having the sequence shown in SEQ ID NO:1 using a GAP algorithm with default parameters, wherein the polynucleotide replicates in a cell.

7. A vector comprising a polynucleotide comprising the sequence shown in SEQ ID NO:1.

8. A polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2-10.

9. A polypeptide comprising an amino acid sequence having at least about 95 % identity to SEQ ID NO:2.

10. A polypeptide comprising an amino acid sequence having at least about 99 % identity to SEQ ID NO:3.

11. A polypeptide comprising an amino acid sequence having at least about 98 % identity to SEQ ID NO:4.

12. A polypeptide comprising an amino acid sequence having at least about 94 % identity to SEQ ID NO:5.

13. A polypeptide comprising an amino acid sequence having at least about 95 % identity to SEQ ID NO:6.

14. A polypeptide comprising an amino acid sequence having at least about 91 % identity to SEQ ID NO:7.

15. A polypeptide comprising an amino acid sequence having at least about 99 % identity to SEQ ID NO:9.

16. A polypeptide comprising an amino acid sequence having at least about 99.5 % identity to SEQ ID NO:10.

17. An antibody that specifically binds a European-like porcine reproductive and respiratory syndrome virus (PRRSV).

18. A method of making an antibody, the method comprising administering to an animal a virus particle comprising an RNA polynucleotide comprising the RNA nucleotide sequence corresponding to SEQ ID NO:1 in an amount effective to cause the production of an antibody specific for the virus particle.

19. The method of claim 18 wherein the antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

20. The method of claim 18 further comprising isolating the antibody.

21. An antibody produced by the method of claim 18.

22. A method of making an antibody, the method comprising administering to an animal a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2-10, or a polynucleotide encoding the polypeptide, in an amount effective to cause the production of an antibody specific for the polypeptide.

23. The method of claim 22 wherein the antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

24. The method of claim 22 further comprising isolating the antibody.

25. An antibody produced by the method of claim 22.

26. A method for detecting a PRRSV, the method comprising:
contacting a virus particle with an antibody of claim 17, 21, or 22 under conditions to form a complex with a virus particle; and
detecting the complex, wherein the presence of the complex indicates the presence of a PRRSV.

27. The method of claim 26 wherein the virus particle is obtained from a biological sample.

28. A method for detecting PRRSV in a porcine subject, comprising:
providing a biological sample from a porcine subject;
adding an antibody of claim 17, 21, or 22 to the sample under conditions to form a complex with a virus particle in the sample; and
detecting the complex, wherein the presence of the complex indicates the presence of PRRSV.

29. The method of claim 28 wherein the biological sample comprises lung tissue.

30. A kit for use in detecting PRRSV in a porcine subject, the kit comprising the antibody of claim 17, 21, or 22 and instructions for using the antibody.

31. A method for detecting the presence of a European-like PRRSV, comprising:
contacting a viral polynucleotide with a first primer and a second primer under conditions suitable to form a detectable amplification product, wherein the first primer comprises a nucleotide sequence that is complementary to nucleotides 2268 and 2269 of SEQ ID NO:1 or the complement thereof; and
detecting an amplification product, wherein the detection indicates that the viral polynucleotide is a European-like PRRSV.

32. The method of claim 31 wherein the first primer comprises a nucleotide sequence selected from the group consisting of 5'ATCGGGAATGCTCAGTCCCCTT (SEQ ID NO:12), and 5'-AAGGGGACTGAGCATTCCCG (SEQ ID NO:14).

33. A method for detecting the presence of a European-like PRRSV in a porcine subject comprising:
contacting a biological sample of a porcine subject with a first primer and a second primer and incubating under conditions suitable to form a detectable amplification product, wherein the first primer comprises a nucleotide sequence that is complementary to nucleotides 2268 and 2269 of SEQ ID NO:1 or the complement thereof; and
detecting an amplification product, wherein the detection indicates that the porcine subject has a European-like PRRSV.

34. The method of claim 33 wherein the first primer comprises a nucleotide sequence selected from the group consisting of 5'ATCGGGAATGCTCAGTCCCCTT (SEQ ID NO:12), and 5'-AAGGGGACTGAGCATTCCCG (SEQ ID NO:14).

35. The method of claim 33 wherein the biological sample comprises lung tissue.

36. A kit for use in detecting PRRSV in a porcine subject, the kit comprising the first primer and a second primer of claim 31 or 33 suitable for use in amplification of a portion of a PRRSV and instructions for using the primer pair.

37. A kit for use in detecting antibody to PRRSV in a porcine subject, the kit comprising the virus of claim 1 and instructions for using the virus.

38. An immunogenic composition comprising an attenuated or inactivated PRRSV comprising a polynucleotide having at least about 96 % identity with a polynucleotide having the sequence shown in SEQ ID NO:1 using a GAP algorithm with default parameters.

39. An immunogenic composition comprising a polypeptide selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, an immunogenic analog thereof, an immunogenic fragment thereof, and a combination thereof.

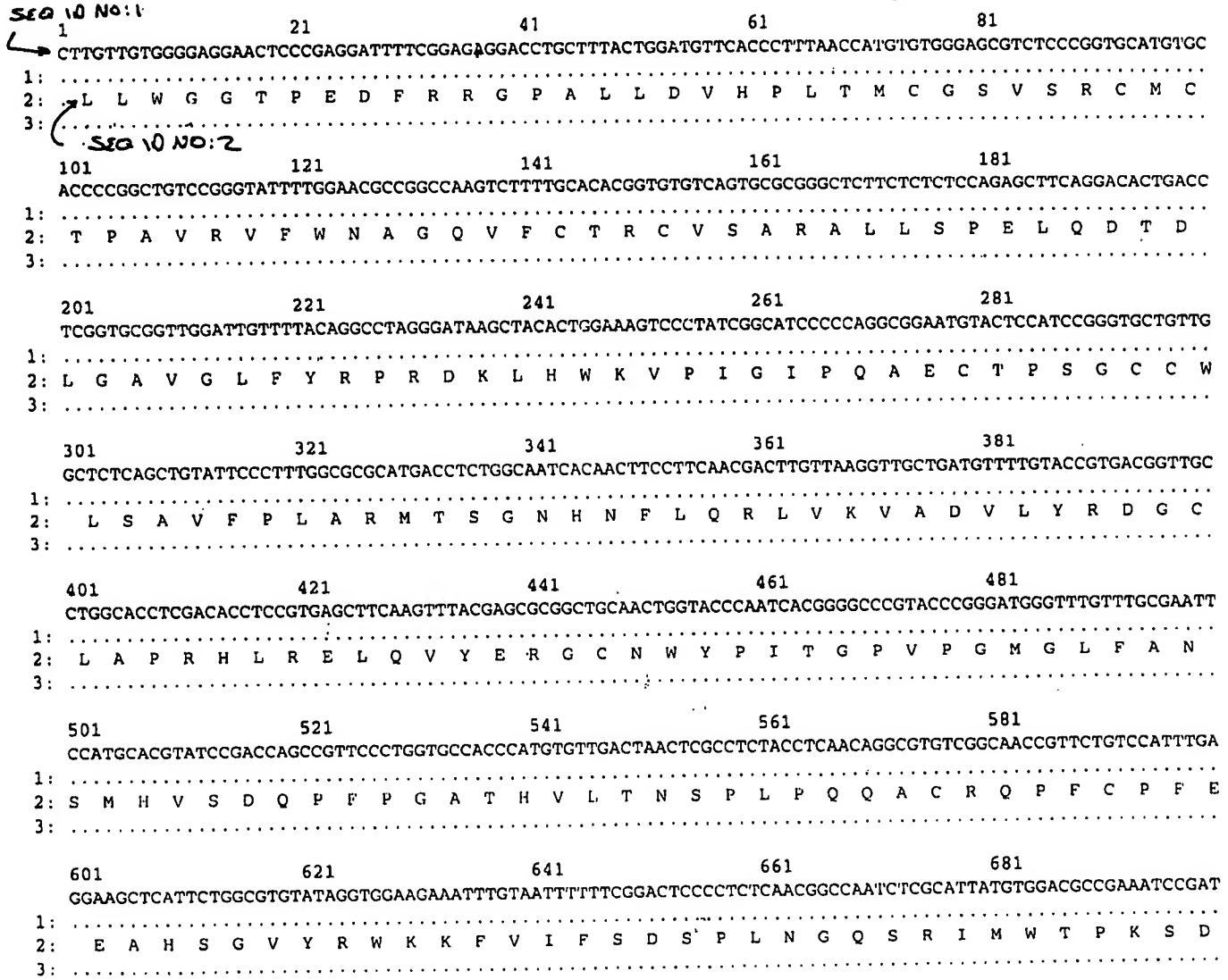
40. A method of treating a porcine subject at risk of infection with a PRRSV or displaying symptoms of a PRRSV infection, comprising administering to the animal an immunogenic composition comprising an attenuated or inactivated PRRSV comprising a polynucleotide having at least about 96 % identity with comprising an RNA polynucleotide comprising the RNA

nucleotide sequence corresponding to SEQ ID NO:1 using a GAP algorithm with default parameters, wherein the immunogenic composition is administered in an amount effective to cause an immune response to the PRRSV.

41. A method of treating a porcine subject at risk of infection with a PRRSV or displaying symptoms of a PRRSV infection, comprising administering to the animal an immunogenic composition comprising a polypeptide selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, an immunogenic analog thereof, an immunogenic fragment thereof, and a combination thereof, wherein the immunogenic composition is administered in an amount effective to cause an immune response to the polypeptide.

42. A method of treating a porcine subject at risk of infection with a PRRSV or displaying symptoms of a PRRSV infection, comprising administering to the animal a neutralizing antibody, wherein the neutralizing antibody is administered in an amount effective to treat the porcine subject.

Fig 1 1A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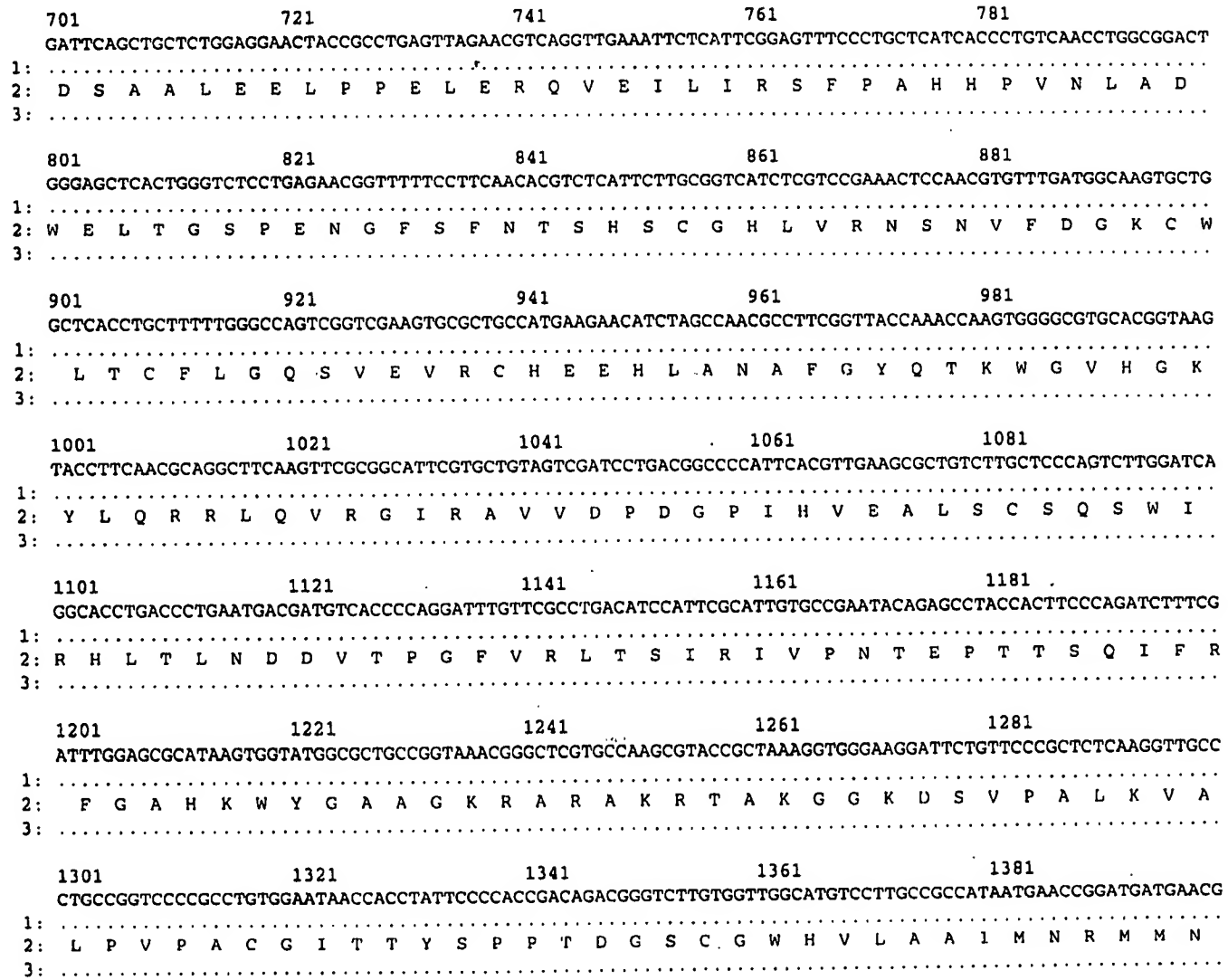
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2: D S A A L E E L P P E L E R Q V E I L I R S F P A H H P V N L A D
3: .....
801 821 841 861 881
GGGAGCTCACTGGGTCTCCTGAGAACGGTTTTTCCTTCAACACGTCTCATTCTTGCGGTCATCTCGTCCGAAACTCCAACGTGTTTGATGGCAAGTGCTG
1: .....
2: W E L T G S P E N G F S F N T S H S C G H L V R N S N V F D G K C W
3: .....
901 921 941 961 981
GCTCACCTGCTTTTTGGGCCAGTCGGTCGAAGTGCGCTGCCATGAAGAACATCTAGCCAACGCCTTCGGTTACCAAACCAAGTGGGGCGTGCACGGTAAG
1: .....
2: L T C F L G Q S V E V R C H E E H L A N A F G Y Q T K W G V H G K
3: .....
1001 1021 1041 1061 1081
TACCTTCAACGCAGGCTTCAAGTTCGCGGCATTCGTGCTGTAGTCGATCCTGACGGCCCCATTCACGTTGAAGCGCTGTCTTGCTCCCAGTCTTGGATCA
1: .....
2: Y L Q R R L Q V R G I R A V V D P D G P I H V E A L S C S Q S W I
3: .....
1101 1121 1141 1161 1181
GGCACCTGACCCTGAATGACGATGTCACCCCAGGATTTGTTCGCCTGACATCCATTCGCATTGTGCCGAATACAGAGCCTACCACTTCCCAGATCTTTCG
1: .....
2: R H L T L N D D V T P G F V R L T S I R I V P N T E P T T S Q I F R
3: .....
1201 1221 1241 1261 1281
ATTTGGAGCGCATAAGTGGTATGGCGCTGCCGGTAAACGGGCTCGTGCCAAGCGTACCGCTAAAGGTGGGAAGGATTCTGTTCCCGCTCTCAAGGTTGCC
1: .....
2: F G A H K W Y G A A G K R A R A K R T A K G G K D S V P A L K V A
3: .....
1301 1321 1341 1361 1381
CTGCCGGTCCCCGCCTGTGGAATAACCACCTATTCCCCACCGACAGACGGGTCTTGTGGTTGGCATGTCCTTGCCGCCATAATGAACCGGATGATGAACG
1: .....
2: L P V P A C G I T T Y S P P T D G S C G W H V L A A I M N R M M N
3: .....

1c

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   1401                1421                1441                1461                1481
   ATGACTTCACGTCCCCTCTGACTCAGTACAACAGACCAGAGGATGATTGGGCTTCAGATTATGATCTTGCTCAGGCGATTCAATGTCTACAACTACCTGC
1: ....................................................................................................
2: D  D  F  T  S  P  L  T  Q  Y  N  R  P  E  D  D  W  A  S  D  Y  D  L  A  Q  A  I  Q  C  L  Q  L  P  A
3: ....................................................................................................

   1501                1521                1541                1561                1581
   TACCGTGGTTCGGAATCGCGCTTGTCCTAACGCCAAGTACCTTATAAAACTTAACGGGGTTCACTGGGAGGTAGAGGTGAGATCTGGAATGGCTCCTCGC
1: ....................................................................................................
2:   T  V  V  R  N  R  A  C  P  N  A  K  Y  L  I  K  L  N  G  V  H  W  E  V  E  V  R  S  G  M  A  P  R
3: ....................................................................................................

   1601                1621                1641                1661                1681
   TCCCTTTCTCGTGAATGTGTGGTCGGCGTTTGTTCTGAAGGCTGCGTCGCGCCGCCTTACCCAGCGGATGGGCTTCCTAAGCGTGCACTCGAGGCCTTGG
1: ....................................................................................................
2:  S  L  S  R  E  C  V  V  G  V  C  S  E  G  C  V  A  P  P  Y  P  A  D  G  L  P  K  R  A  L  E  A  L
3: ....................................................................................................

   1701                1721                1741                1761                1781
   CGTCTGCTTACAGACTACCCTCCGATTGTGTTTGCTCTGGTATTGCTGACTTTCTTGCCAATCCACCTCCTCAAGAATTCTGGACTCTCGACAAAATGTT
1: ....................................................................................................
2: A  S  A  Y  R  L  P  S  D  C  V  C  S  G  I  A  D  F  L  A  N  P  P  P  Q  E  F  W  T  L  D  K  M  L
3: ....................................................................................................

   1801                1821                1841                1861                1881
   GACCTCCCCGTCACCAGAACGGTCCGGCTTCTCTAGTTTGTATAACTTGCTATTAGAGGTTGTTCCGCAAAAATGCGGTGTCACGGAAGGGGCCTTCACC
1: ....................................................................................................
2:   T  S  P  S  P  E  R  S  G  F  S  S  L  Y  N  L  L  L  E  V  V  P  Q  K  C  G  V  T  E  G  A  F  T
3: ....................................................................................................

   1901                1921                1941                1961                1981
   TATGCTGTTGAGAGGATGTTAATGGATTGTCCGAGCTCCGAACAGGCCATGGCTCTTCTGGCAAAAATTAAAGTTCCATCCTCAAAGGCCCCATCTGTGT
1: ....................................................................................................
2:  Y  A  V  E  R  M  L  M  D  C  P  S  S  E  Q  A  M  A  L  L  A  K  I  K  V  P  S  S  K  A  P  S  V
3: ....................................................................................................

   2001                2021                2041                2061                2081
   CCTTGGACGAGTGTTTCCCTGCAGATGTTCCGGCCGATTTCGAGCCAACGTCTCAGAAAAGGCCCCAAAGTTCCGGCGCCGCTGTCGCCCTGTGTTCATC
1: ....................................................................................................
2: S  L  D  E  C  F  P  A  D  V  P  A  D  F  E  P  T  S  Q  K  R  P  Q  S  S  G  A  A  V  A  L  C  S  S
3: ....................................................................................................
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      2101        2121        2141        2161        2181
      GGATGCAGAAGGGTTCGAGGAAGCAGCCCCAGAAGGAGTTCAAGAGAGAGGCCATAAGGCCGTCCACTCTGCACTCTTTGCCAAGGGTCCAAATAACGAA
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:      D  A  E  G  F  E  E  A  A  P  E  G  V  Q  E  R  G  H  K  A  V  H  S  A  L  F  A  K  G  P  N  N  E
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........

      2201        2221        2241        2261        2281
      CAGGTACAGGTGGTTGCCGGTGAGCAACAGAAGCTCGGCGGTTGTGGTTTGGCAATCGGGAATGCTCAGTCCCCTTTAAATTCCATGAAAGAAAACATGC
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:    Q  V  Q  V  V  A  G  E  Q  Q  K  L  G  G  C  G  L  A  I  G  N  A  Q  S  P  L  N  S  M  K  E  N  M
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........

      2301        2321        2341        2361        2381
      GCAGTAGCCGGGAAGACGAACCACTGGATTTGTCCCAACCAGCACCAGTTGCCGCAACGACCCTTGAGAGAGAGCAAACACCGATAACCCAGGTTCTGA
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:    R  S  S  R  E  D  E  P  L  D  L  S  Q  P  A  P  V  A  A  T  T  L  E  R  E  Q  T  P  D  N  P  G  S  D
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........

      2401        2421        2441        2461        2481
      TGCCGGTGCCCTCCCCGCCACCGTTCGAGAATCTGTCCCGACAGGCCTATGCTCCGTCATGTTGAGCACTGTGGCACGGAGTCTGGCGATAGCAGTTCG
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:      A  G  A  L  P  A  T  V  R  E  S  V  P  T  G  P  M  L  R  H  V  E  H  C  G  T  E  S  G  D  S  S  S
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........

      2501        2521        2541        2561        2581
      CCTTTGGATCTGTCTTATGCGCAAACTTTGGACCAGCCTTTAGATCTATCCCTGGCCGTTTGGCCGGTGAAGGCCACCGCGTCTGACCCTGGCTGGGTCC
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:    P  L  D  L  S  Y  A  Q  T  L  D  Q  P  L  D  L  S  L  A  V  W  P  V  K  A  T  A  S  D  P  G  W  V
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........

      2601        2621        2641        2661        2681
      ACGGTAGGCGCGAGCCTGTCTTTGTAAAGCCTCGAAAAGCTTTCTCTGATAGCGACTCAGCCTTTCAGTTCGGGAAGCTTTCTGAGTCCGGCTCTGTCAT
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:    H  G  R  R  E  P  V  F  V  K  P  R  K  A  F  S  D  S  D  S  A  F  Q  F  G  K  L  S  E  S  G  S  V  I
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........

      2701        2721        2741        2761        2781
      TGAGTTTGACCGAACAAAAGATGCTCCGGTGGTTGACGCCCCCTGTTGGCTCGACGACTTCGAACGAGGCACTGTCTATAGCCGATCCTTTCGAATTTGCC
1:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
2:      E  F  D  R  T  K  D  A  P  V  V  D  A  P  V  G  S  T  T  S  N  E  A  L  S  I  A  D  P  F  E  F  A
3:    ..........*.........*.........*.........*.........*.........*.........*.........*.........*.........
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Ie

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   2801                2821                2841                2861                2881
   GAACTCAAGCGCCCGCGTTTCTCCGCACAAGCCTTAATTGACCGAGGCGGTCCGCTTGCCGATGTCCATGCGAAAATAAAGAACCGGGTGTATGAACGGT
1: ....................................................................................................
2:  E  L  K  R  P  R  F  S  A  Q  A  L  I  D  R  G  G  P  L  A  D  V  H  A  K  I  K  N  R  V  Y  E  R
3: ....................................................................................................

   2901                2921                2941                2961                2981
   GCCTCCAAGCTTGTGAGCCCGGTAGTCGTGCAACCCCAGCCACCAAGGAGTGGCTCGACAAGATGTGGGATAGGGTGGACATGAAAACTTGGTGCTGCAC
1: ....................................................................................................
2: C  L  Q  A  C  E  P  G  S  R  A  T  P  A  T  K  E  W  L  D  K  M  W  D  R  V  D  M  K  T  W  C  C  T
3: ....................................................................................................

   3001                3021                3041                3061                3081
   CTCGCAGTTCCAAGCTGGTCGCATTCTTGCGTCCCTCAAATTCCTCCCTGACATGATTCAAGACACACCGCCTCCTGTTCCCAGGAAGAACCGAGCTAGT
1: ....................................................................................................
2:   S  Q  F  Q  A  G  R  I  L  A  S  L  K  F  L  P  D  M  I  Q  D  T  P  P  P  V  P  R  K  N  R  A  S
3: ....................................................................................................

   3101                3121                3141                3161                3181
   GACAATGCCGATCTGAAGCAACTGGTGGCACAGTGGGATAGGAAATTGAGTATGACCCCTCCCCAAAAACCGGTTGAGCCAGTGCTTGACCAGACCGTCT
1: ....................................................................................................
2:  D  N  A  D  L  K  Q  L  V  A  Q  W  D  R  K  L  S  M  T  P  P  Q  K  P  V  E  P  V  L  D  Q  T  V
3: ....................................................................................................

   3201                3221                3241                3261                3281
   CTCCGCCTACGGATACTCAGCAAGAAGATGTGACCCCCTCCGATGGGCCACCCCATGCGCCGGATTTTCCCAGTCGTGTGAGCACGGGCGGGAGTTGGAA
1: ....................................................................................................
2: S  P  P  T  D  T  Q  Q  E  D  V  T  P  S  D  G  P  P  H  A  P  D  F  P  S  R  V  S  T  G  G  S  W  K
3: ....................................................................................................

   3301                3321                3341                3361                3381
   AGACCTTATGTGTTCCGGCACCCGTCTCGCGGGGTCTATCAGTCAGCGCCTCATGACATGGGTTTTTGAAGTTTTCTCCCACCTCCCAGCCTTTATGCTC
1: ....................................................................................................
2:   D  L  M  C  S  G  T  R  L  A  G  S  I  S  Q  R  L  M  T  W  V  F  E  V  F  S  H  L  P  A  F  M  L
3: ....................................................................................................

   3401                3421                3441                3461                3481
   ACACTTTTCTCGCCGCGGGGCTCTATGGCTCCAGGTGATTGGTTGTTTGCAGGTGTTGTTTTACTTGCTCTCTTGCTCTGTCATTCTTACCCGATACTCG
1: ....................................................................................................
2:  T  L  F  S  P  R  G  S  M  A  P  G  D  W  L  F  A  G  V  V  L  L  A  L  L  L  C  H  S  Y  P  I  L
3: ....................................................................................................
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1f

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   3501                3521                3541                3561                3581
   GGTGCCTTCCCTTATTGGGTGTCTTTTCTGGTTCTTTGCGGCGTGTTCGTCTGGGTGTTTTTGGTTCTTGGATGGCTTTTGCTGTATTTTTATTCTCGAC
1: ..................................................................................................
2: G  C  L  P  L  L  G  V  F  S  G  S  L  R  R  V  R  L  G  V  F  G  S  W  M  A  F  A  V  F  L  F  S  T
3: ..................................................................................................

   3601                3621                3641                3661                3681
   TCCATCCAACCCAGTCGGTTCTTCTTGTGACCACGATTCGCCGGAATGTCATGCTGAGCTTTTGGCTCTTGAGCAGCGCCAACTTTGGGAACCTGTGCGC
1: ..................................................................................................
2:   P  S  N  P  V  G  S  S  C  D  H  D  S  P  E  C  H  A  E  L  L  A  L  E  Q  R  Q  L  W  E  P  V  R
3: ..................................................................................................

   3701                3721                3741                3761                3781
   GGCCTTGTGGTCGGCCCCTCGGGCCTCTTATGTGTCATTCTTGGCAAGTTACTCGGTGGGTCACGTTATCTCTGGCATATTCTCCTACGTTTATGCATGC
1: ..................................................................................................
2:  G  L  V  V  G  P  S  G  L  L  C  V  I  L  G  K  L  L  G  G  S  R  Y  L  W  H  I  L  L  R  L  C  M
3: ..................................................................................................

   3801                3821                3841                3861                3881
   TTACAGATTTGGCCCTTTCTCTTGTTTATGTGGTGTCCCAAGGGCGTTGTCACAAGTGTTGGGGAAAGTGTATAAGAACAGCTCCTACTGAGGTGGCTCT
1: ..................................................................................................
2: L  T  D  L  A  L  S  L  V  Y  V  V  S  Q  G  R  C  H  K  C  W  G  K  C  I  R  T  A  P  T  E  V  A  L
3: ..................................................................................................

   3901                3921                3941                3961                3981
   TAATGTGTTTCCTTTCACGCGCGCCACCCGTTCCTCTCTTGTATCCTTGTGTGATCGATTCCAAACGCCAAAAGGGGTTGATCCTGTGCACTTGGCAACG
1: ..................................................................................................
2:   N  V  F  P  F  T  R  A  T  R  S  S  L  V  S  L  C  D  R  F  Q  T  P  K  G  V  D  P  V  H  L  A  T
3: ..................................................................................................

   4001                4021                4041                4061                4081
   GGTTGGCGCGGGTGCTGGCGTGGTGGGAGTCCCGTCCATCAACCACACCAAAAGCCCATTGCTTATGCCAACTTGGATGAAAAGAAAATATCTGCCCAAA
1: ..................................................................................................
2:  G  W  R  G  C  W  R  G  G  S  P  V  H  Q  P  H  Q  K  P  I  A  Y  A  N  L  D  E  K  K  I  S  A  Q
3: ..................................................................................................

   4101                4121                4141                4161                4181
   CGGTGGTTGCCGTCCCATACGATCCCAGTCAGGCCATCAAATGCCTGAAAGTTCTGCAGGCGGGAGGGGCTATCGTGGACCAGCCTACACCTGAGGTTGT
1: ..................................................................................................
2: T  V  V  A  V  P  Y  D  P  S  Q  A  I  K  C  L  K  V  L  Q  A  G  G  A  I  V  D  Q  P  T  P  E  V  V
3: ..................................................................................................
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4201                4221                4241                4261                4281
TCGTGTGTCCGAAATCCCCTTCTCAGCTCCATTTTTTCCAAAAGTTCCAGTCAACCCAGATTGCAGAGTCGTGGTAGATTCGGACACTTTTGTGGCTGCG
1: .................................................................................................
2:  R  V  S  E  I  P  F  S  A  P  F  F  P  K  V  P  V  N  P  D  C  R  V  V  V  D  S  D  T  F  V  A  A
3: .................................................................................................

4301                4321                4341                4361                4381
GTTCGATGCGGTTACTCGACAGCACAACTGGTCTTGGGCCAGGGCAACTTTGCCAAGTTGAATCAGACCCCCCCCAGGAACTCCACTTCCACCAAAACGA
1: .................................................................................................
2:  V  R  C  G  Y  S  T  A  Q  L  V  L  G  Q  G  N  F  A  K  L  N  Q  T  P  P  R  N  S  T  S  T  K  T
3: .................................................................................................

4401                4421                4441                4461                4481
CTGGTGGGGCCTCTTACACCCTTGCTGTGGCTCAAGTAACTGTGTGGACTCTTTTTCATTTCATCCTCGGCCTTTGGTTTACATCACCTCAAGTGTGTGG
1: .................................................................................................
2: T  G  G  A  S  Y  T  L  A  V  A  Q  V  T  V  W  T  L  F  H  F  I  L  G  L  W  F  T  S  P  Q  V  C  G
3: .................................................................................................

4501                4521                4541                4561                4581
CCGAGGAACCGCTGACCCATGGTGTTCAAATCCTTTTTCATACCCCACCTATGGCCCTGGAGTTGTGTGCTCCTCTCGGCTTTGCGTGTCTGCCGACGGG
1: .................................................................................................
2:  R  G  T  A  D  P  W  C  S  N  P  F  S  Y  P  T  Y  G  P  G  V  V  C  S  S  R  L  C  V  S  A  D  G
3: .................................................................................................

4601                4621                4641                4661                4681
GTCACCTTGCCATTGTTCTCAGCCGTGGCACAACTTTCCGGTAGAGAGGTGGGGATCTTTATTTGGTGCTCGTTTCCTTGATTGCCTTGGCCCACCGTA
1: .................................................................................................
2:  V  T  L  P  L  F  S  A  V  A  Q  L  S  G  R  E  V  G  I  F  I  L  V  L  V  S  L  I  A  L  A  H  R
3: .................................................................................................

4701                4721                4741                4761                4781
TGGCTCTTAAGGCAGACATGTTAGTCGTCTTTTTGGCTTTTTGTGCTTACGCCTGGCCTATGAGCTCCTGGTTAATTTGCTTCTTTCCCTTACTCTTGAA
1: .................................................................................................
2: M  A  L  K  A  D  M  L  V  V  F  L  A  F  C  A  Y  A  W  P  M  S  S  W  L  I  C  F  F  P  L  L  L  K
3: .................................................................................................

4801                4821                4841                4861                4881
GTGGGTCACCCTTCACCCTCTCACCATGCTTTGGGTGCACTCATTCTTGGTGTTTTGTCTGCCAGCGGCCGGCATCCTCTCATTAGGGACAACTGGCCTT
1: .................................................................................................
2:  W  V  T  L  H  P  L  T  M  L  W  V  H  S  F  L  V  F  C  L  P  A  A  G  I  L  S  L  G  T  T  G  L
3: .................................................................................................
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   4901                4921                4941                4961                4981
   CTCTGGGCAATTGGCCGCTTTACCCAGGTTGCCGGAATTATTACACCTTATGACATCCACCAATACACCTCTGGGCCACGTGGTGCAGCTGCTGTGGCCA
1: ..................................................................................................
2:  L  W  A  I  G  R  F  T  Q  V  A  G  I  I  T  P  Y  D  I  H  Q  Y  T  S  G  P  R  G  A  A  A  V  A
3: ..................................................................................................

   5001                5021                5041                5061                5081
   CAGCCCCAGAAGGCACTTATATGGCCGCCGTCCGGAGAGCTGCTTTAACTGGGCGAACTTTAATCTTCACCCCGTCTGCAGTTGGATCCCTTCTCGAAGG
1: ..................................................................................................
2: T  A  P  E  G  T  Y  M  A  A  V  R  R  A  A  L  T  G  R  T  L  I  F  T  P  S  A  V  G  S  L  L  E  G
3: ..................................................................................................

   5101                5121                5141                5161                5181
   TGCCTTCAGGACTCAAAAACCCTGCCTTAACACCGTGAATGTTGTAGGCTCTTCCCTTGGTTCCGGAGGGGTTTTCACCATTAACGGCAGAAGGACTGTC
1: ..................................................................................................
2:   A  F  R  T  Q  K  P  C  L  N  T  V  N  V  V  G  S  S  L  G  S  G  G  V  F  T  I  N  G  R  R  T  V
3: ..................................................................................................

   5201                5221                5241                5261                5281
   GTCACTGCTGCTCATGTGTTGAACGGTGACACAGCTAGAGTCACCGGCGACTCCTACAACCGCATGCACACTTTCAAGACCAATGGTGATTATGCCTGGT
1: ..................................................................................................
2:  V  T  A  A  H  V  L  N  G  D  T  A  R  V  T  G  D  S  Y  N  R  M  H  T  F  K  T  N  G  D  Y  A  W
3: ..................................................................................................

   5301                5321                5341                5361                5381
   CCCATGCTGATGACTGGCAGGGCGTTGCTCCTGTGGTCAAGGTTGCGAAGGGGTACCGCGGTCGTGCCTACTGGCAAACATCAACTGGTGTCGAACCCGG
1: ..................................................................................................
2: S  H  A  D  D  W  Q  G  V  A  P  V  V  K  V  A  K  G  Y  R  G  R  A  Y  W  Q  T  S  T  G  V  E  P  G
3: ..................................................................................................

   5401                5421                5441                5461                5481
   CATTATTGGGGAAGGGTTCGCCTTCTGTTTCACCAACTGTGGCGATTCAGGGTCACCTGTTATCTCAGAATCTGGTGATCTCATTGGAATCCACACTGGT
1: ..................................................................................................
2:   I  I  G  E  G  F  A  F  C  F  T  N  C  G  D  S  G  S  P  V  I  S  E  S  G  D  L  I  G  I  H  T  G
3: ..................................................................................................

   5501                5521                5541                5561                5581
   TCAAACAAACTCGGTTCTGGTCTTGTGACGACCCCTGAAGGGGAGACCTGCGCCATCAAAGAAACCAAGCTCTCTGACCTTTCCAGACATTTTGCAGGCC
1: ..................................................................................................
2:  S  N  K  L  G  S  G  L  V  T  T  P  E  G  E  T  C  A  I  K  E  T  K  L  S  D  L  S  R  H  F  A  G
3: ..................................................................................................
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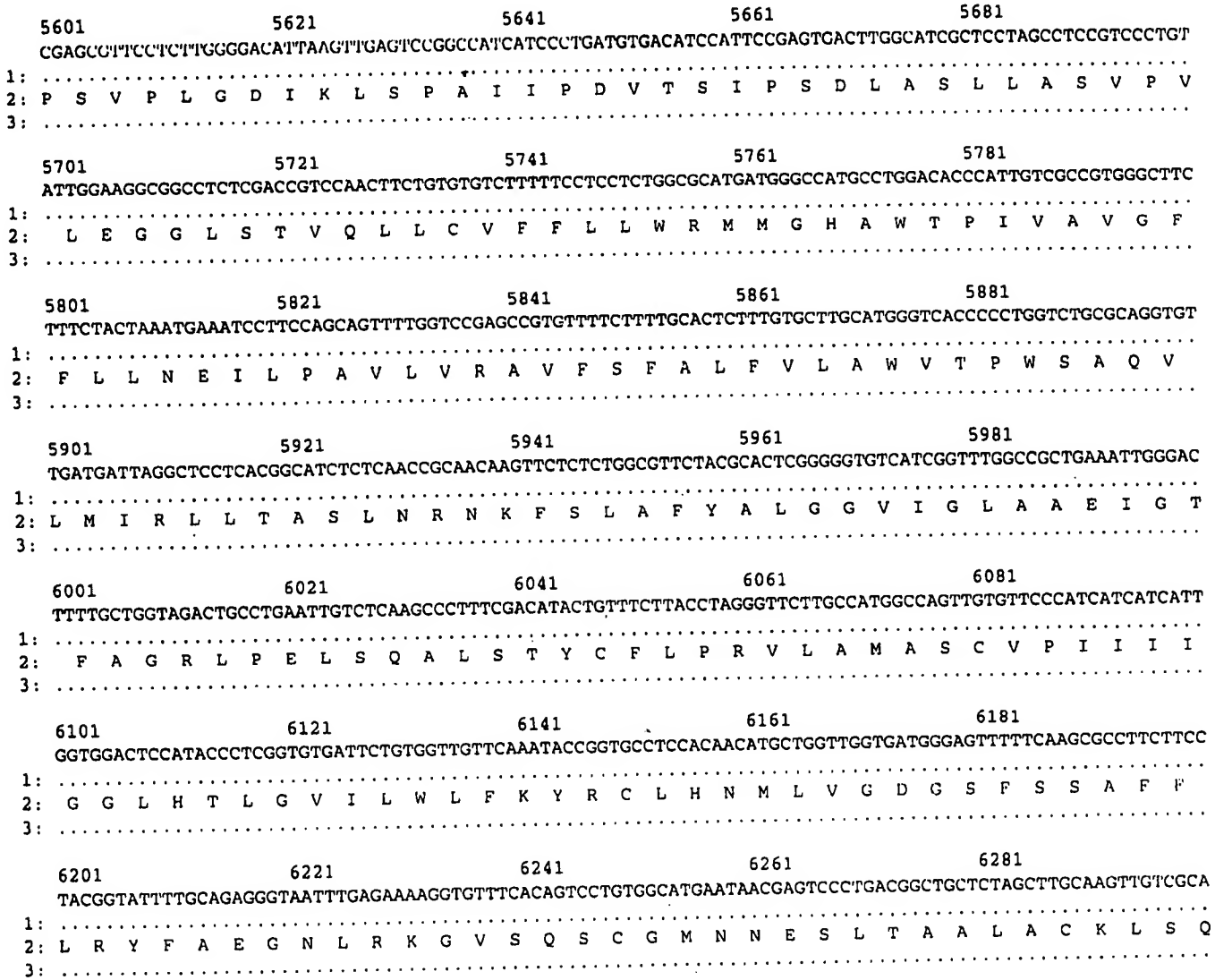
5601 5621 5641 5661 5681
CGAGCGTTCCTCTTGGGGACATTAAGTTGAGTCCGGCCATCATCCCTGATGTGACATCCATTCCGAGTGACTTGGCATCGCTCCTAGCCTCCGTCCCTGT
1: .....
2: P S V P L G D I K L S P A I I P D V T S I P S D L A S L L A S V P V
3: .....
5701 5721 5741 5761 5781
ATTGGAAGGCGGCCTCTCGACCGTCCAACTTCTGTGTGTCTTTTTCCTCCTCTGGCGCATGATGGGCCATGCCTGGACACCCATTGTCGCCGTGGGCTTC
1: .....
2: L E G G L S T V Q L L C V F F L L W R M M G H A W T P I V A V G F
3: .....
5801 5821 5841 5861 5881
TTTCTACTAAATGAAATCCTTCCAGCAGTTTTGGTCCGAGCCGTGTTTTCTTTTGCACTCTTTGTGCTTGCATGGGTCACCCCCTGGTCTGCGCAGGTGT
1: .....
2: F L L N E I L P A V L V R A V F S F A L F V L A W V T P W S A Q V
3: .....
5901 5921 5941 5961 5981
TGATGATTAGGCTCCTCACGGCATCTCTCAACCGCAACAAGTTCTCTCTGGCGTTCTACGCACTCGGGGGTGTCATCGGTTTGGCCGCTGAAATTGGGAC
1: .....
2: L M I R L L T A S L N R N K F S L A F Y A L G G V I G L A A E I G T
3: .....
6001 6021 6041 6061 6081
TTTTGCTGGTAGACTGCCTGAATTGTCTCAAGCCCTTTCGACATACTGTTTCTTACCTAGGGTTCTTGCCATGGCCAGTTGTGTTCCCATCATCATCATT
1: .....
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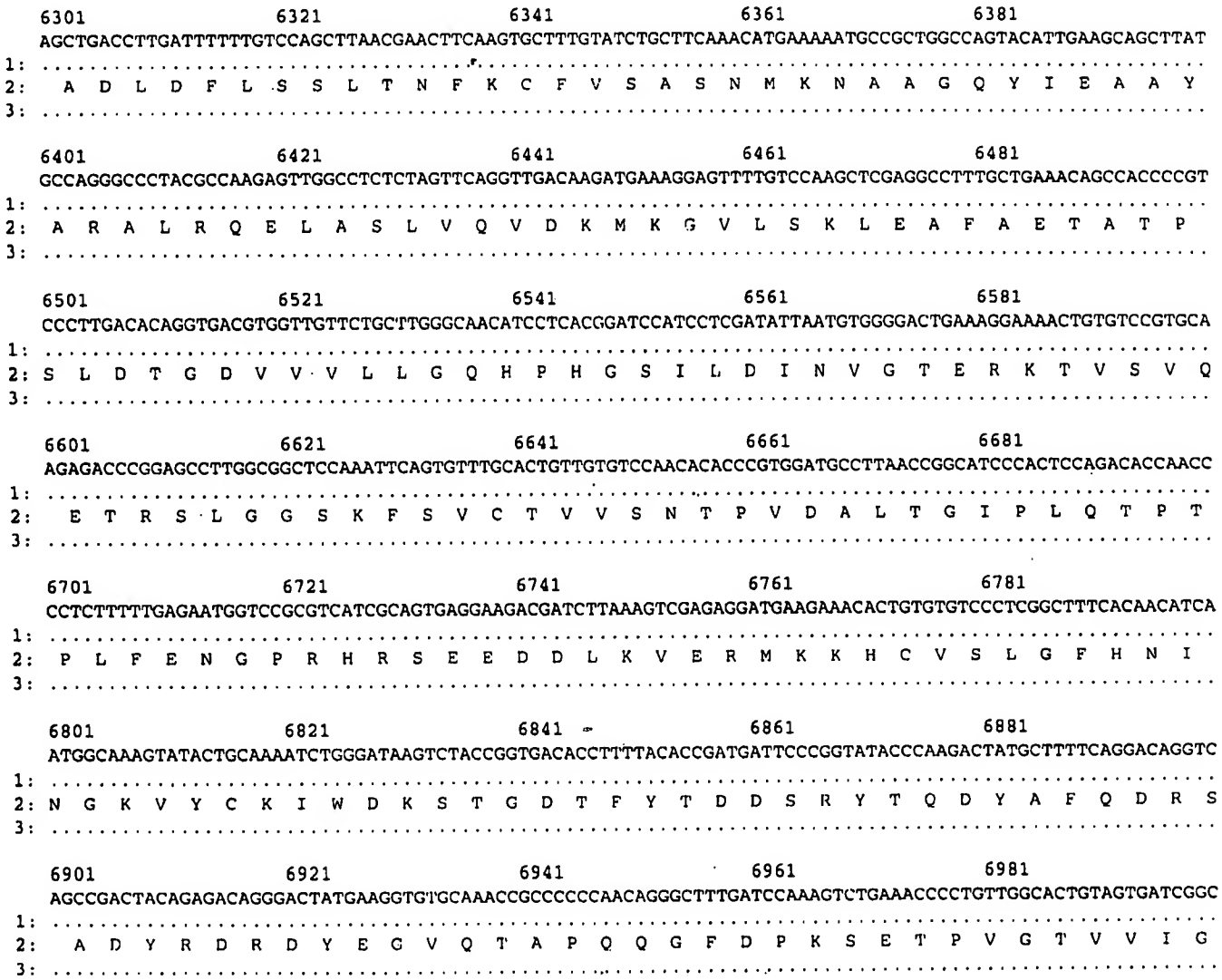
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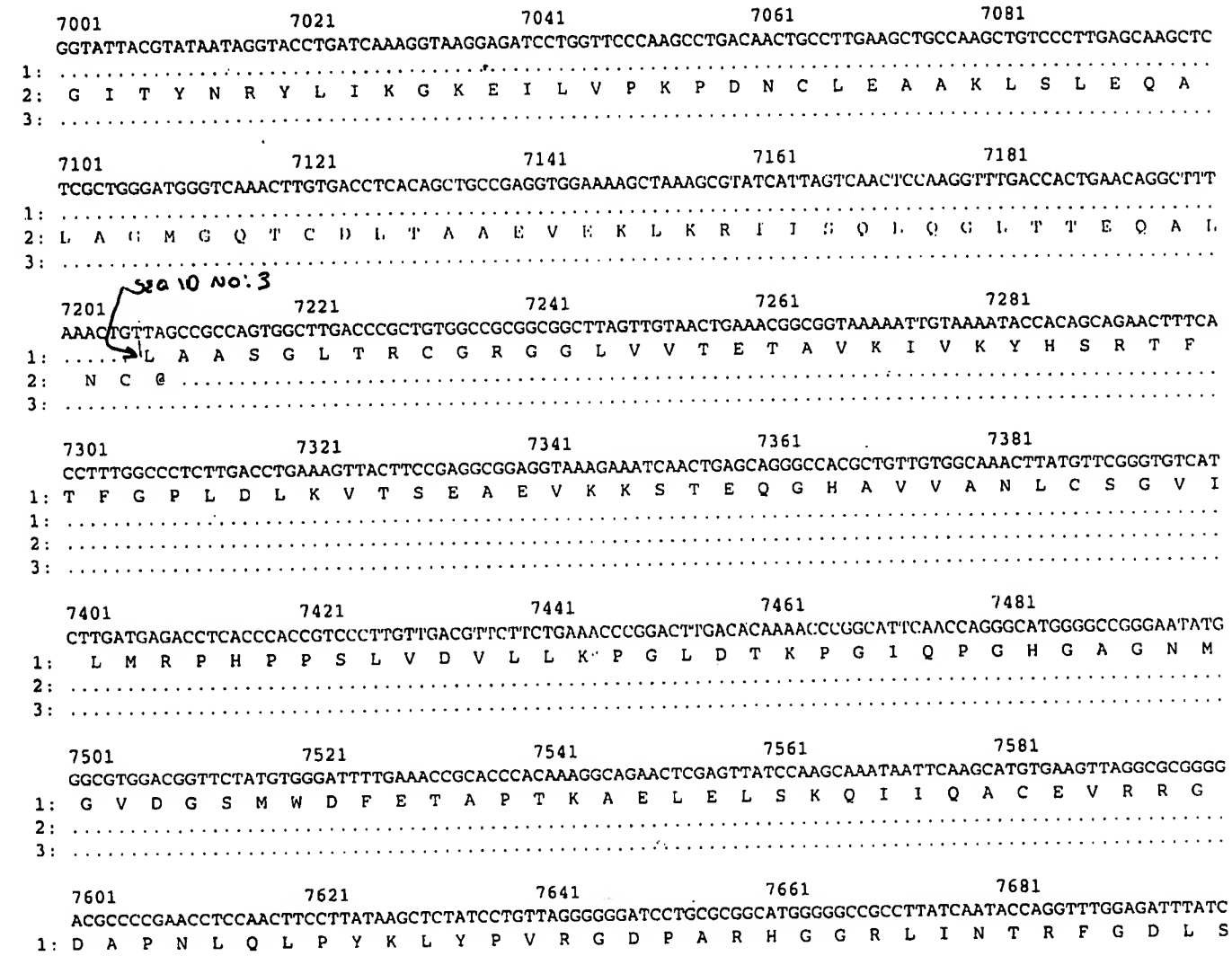
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3:
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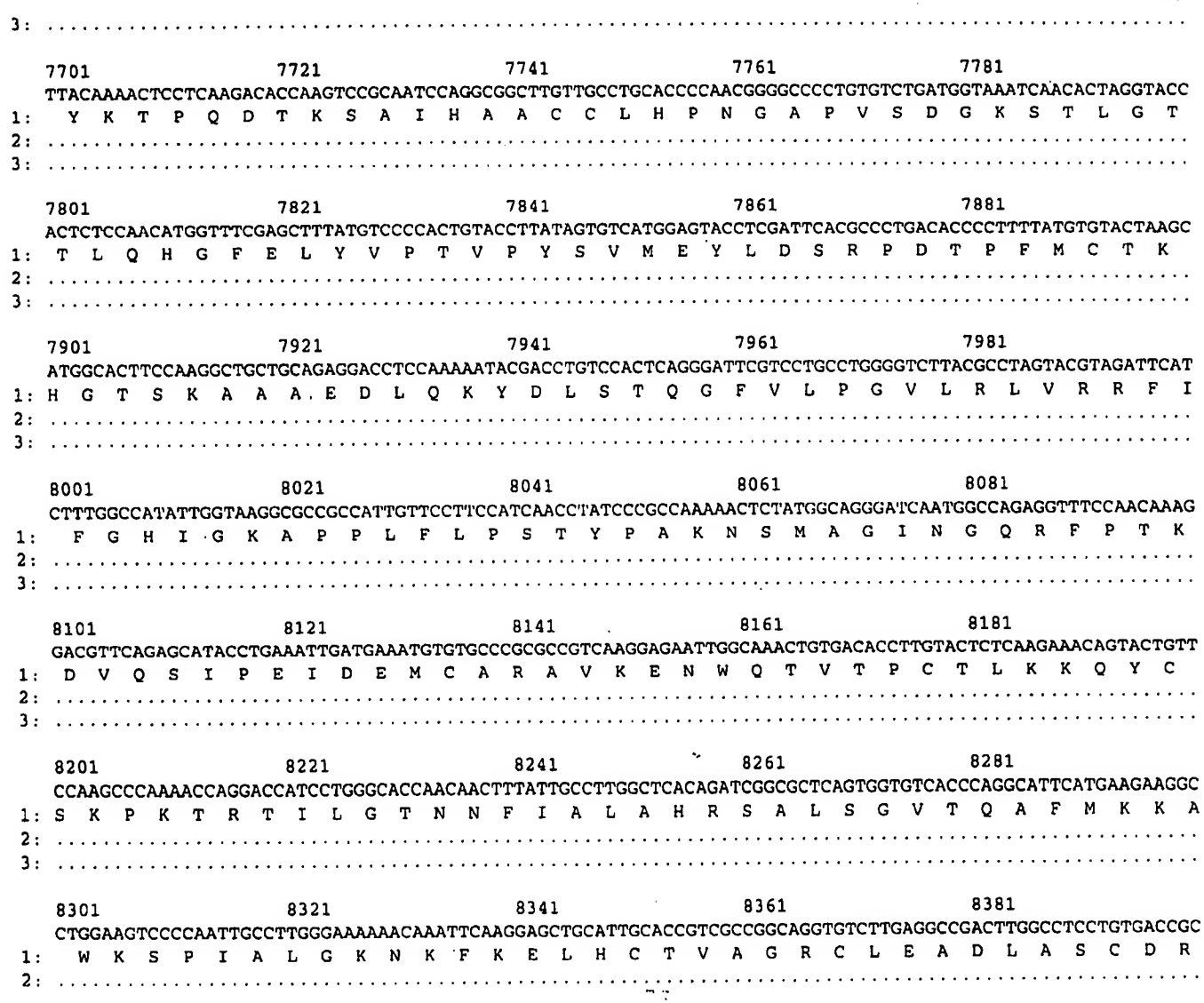
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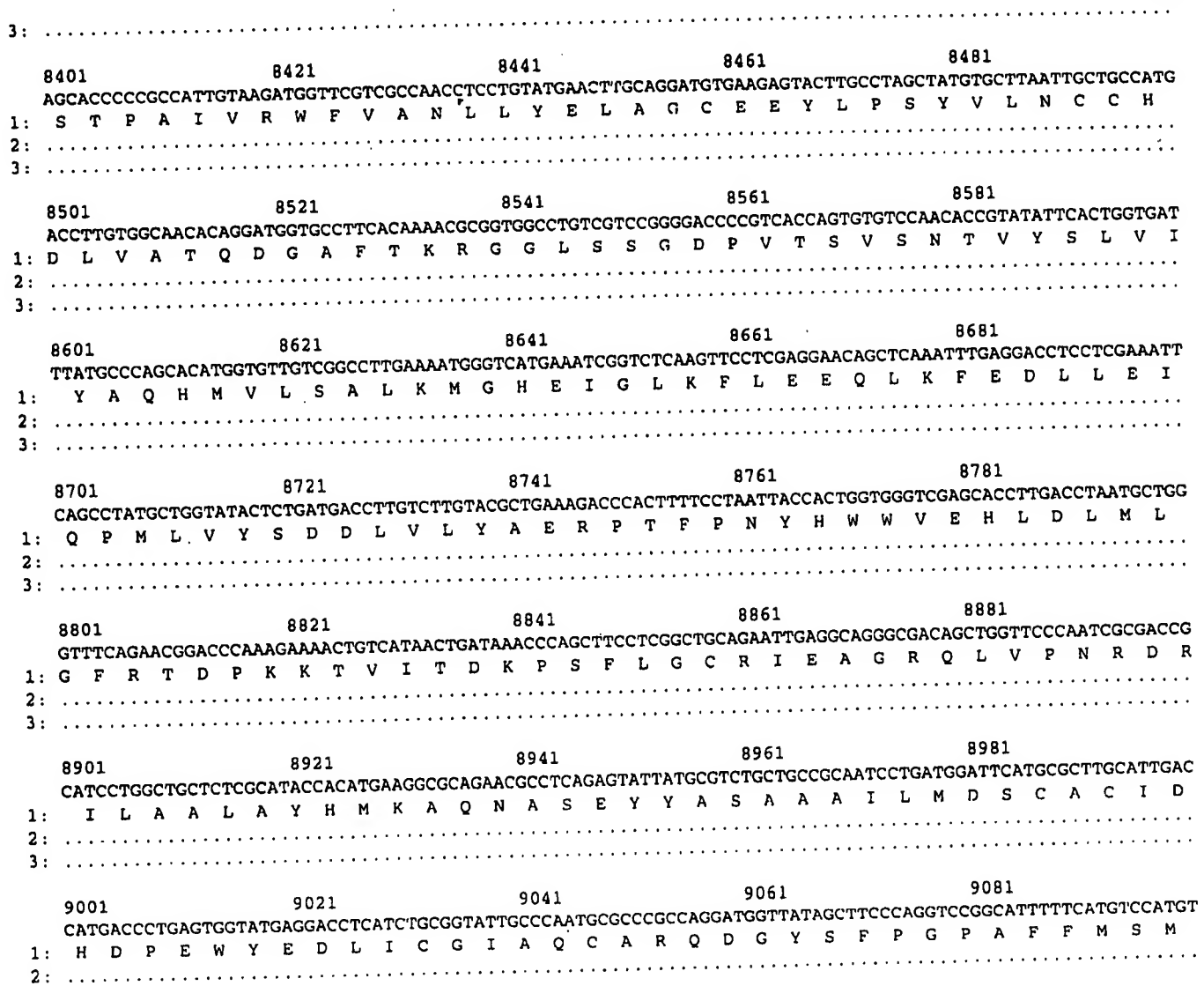
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3: .....
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1: .....
2: .....

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3: ..........

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2: ..........
3: ..........

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2: ..........
3: ..........

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2: ..........
3: ..........

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2: ..........
3: ..........

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2: ..........
3: ..........

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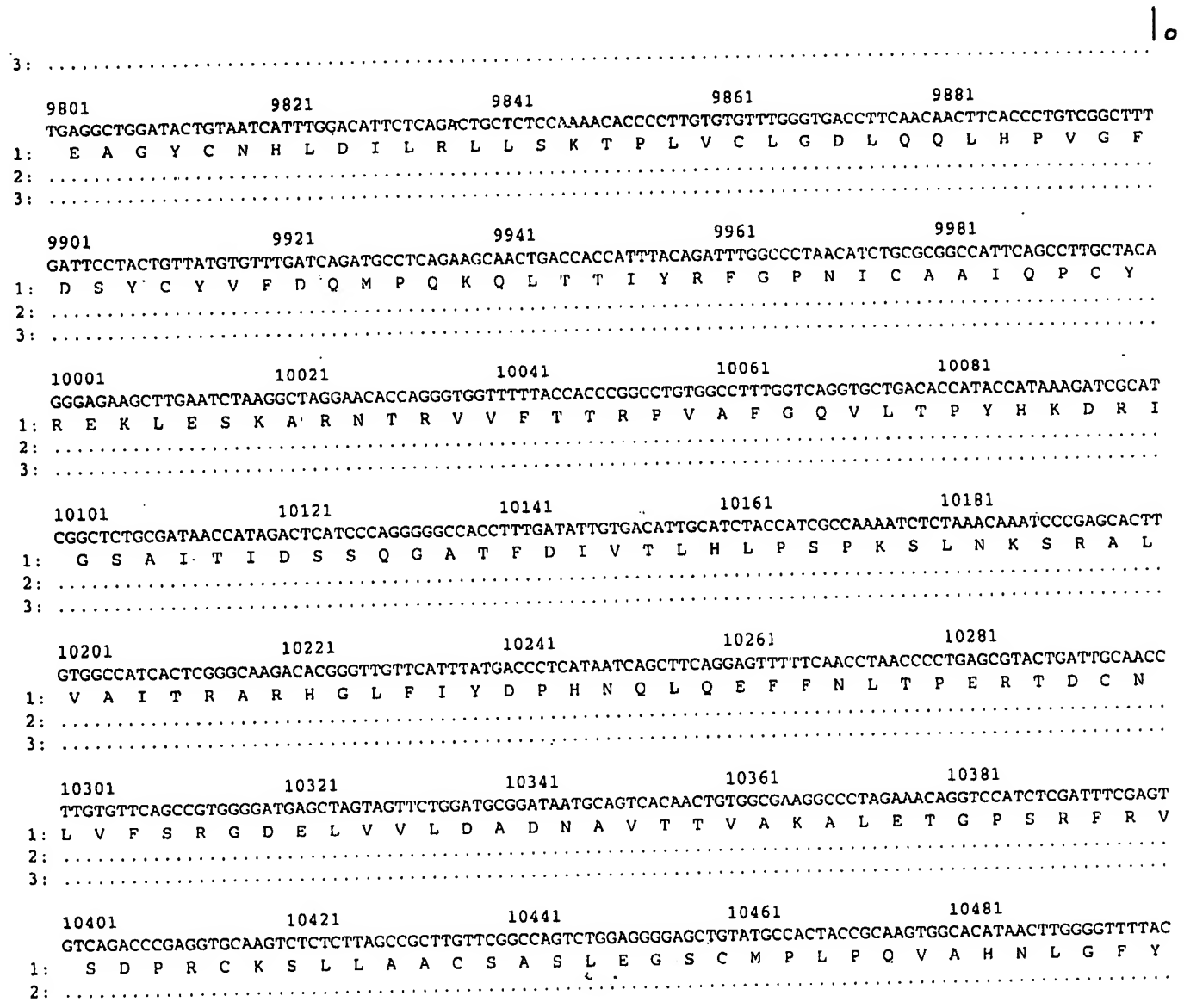
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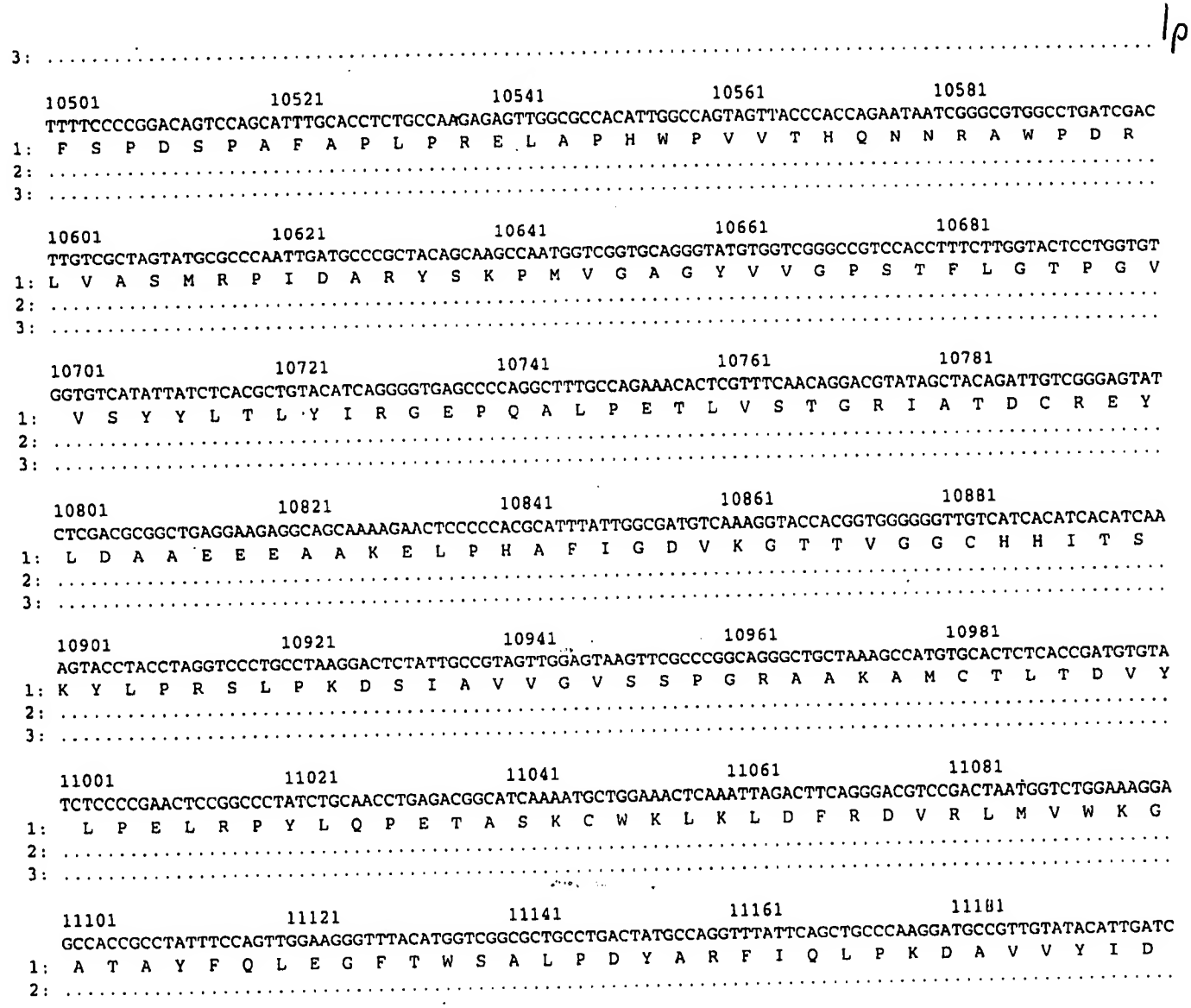
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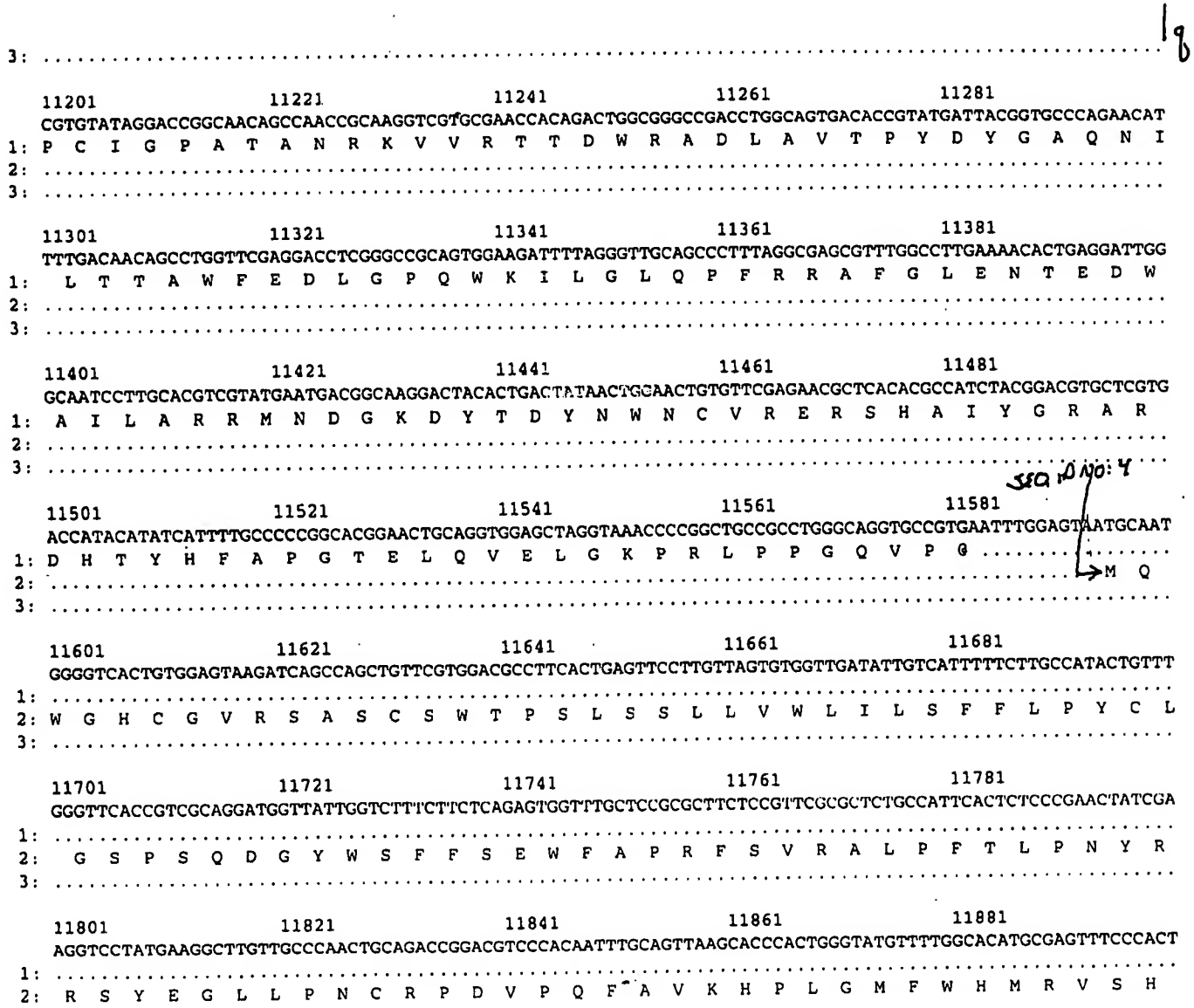
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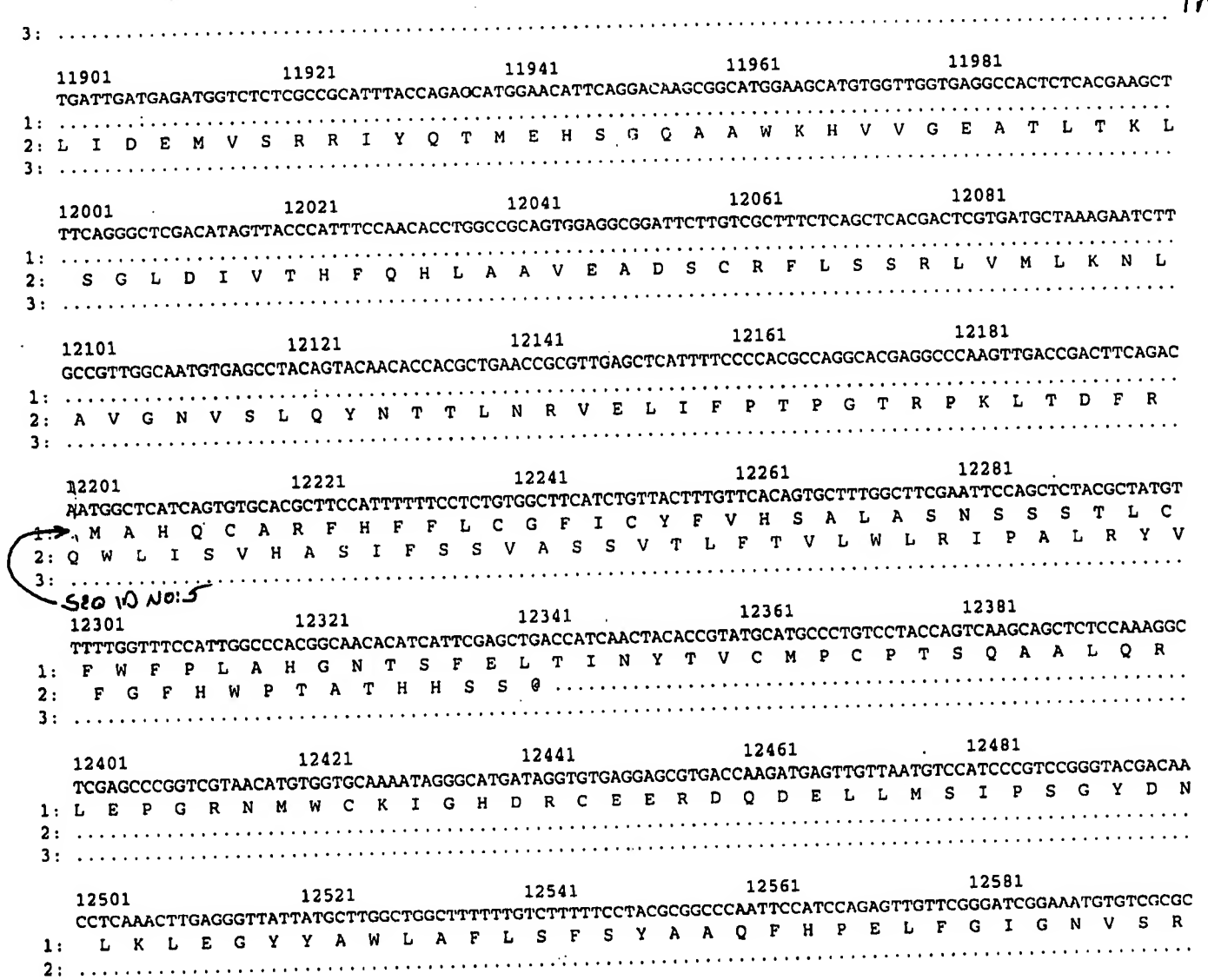
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2: F G F H W P T A T H H S S @
12401 12421 12441 12461 12481
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1: L E P G R N M W C K I G H D R C E E R D Q D E L L M S I P S G Y D N
12501 12521 12541 12561 12581
CCTCAAACTTGAGGGTTATTATGCTTGGCTGGCTTTTTTGTCTTTTTCCTACGCGGCCCAATTCCATCCAGAGTTGTTCGGGATCGGAAATGTGTCGCGC
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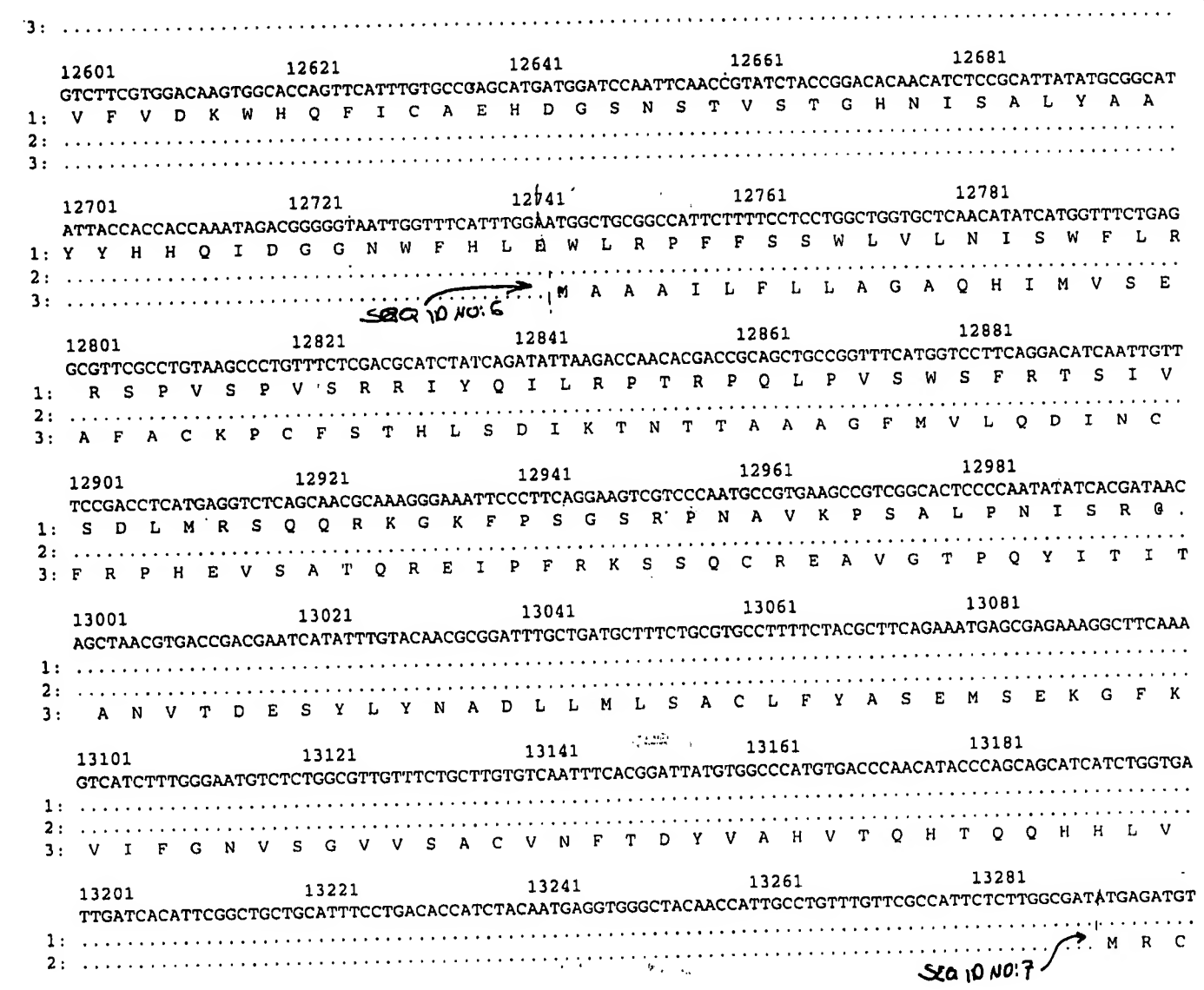
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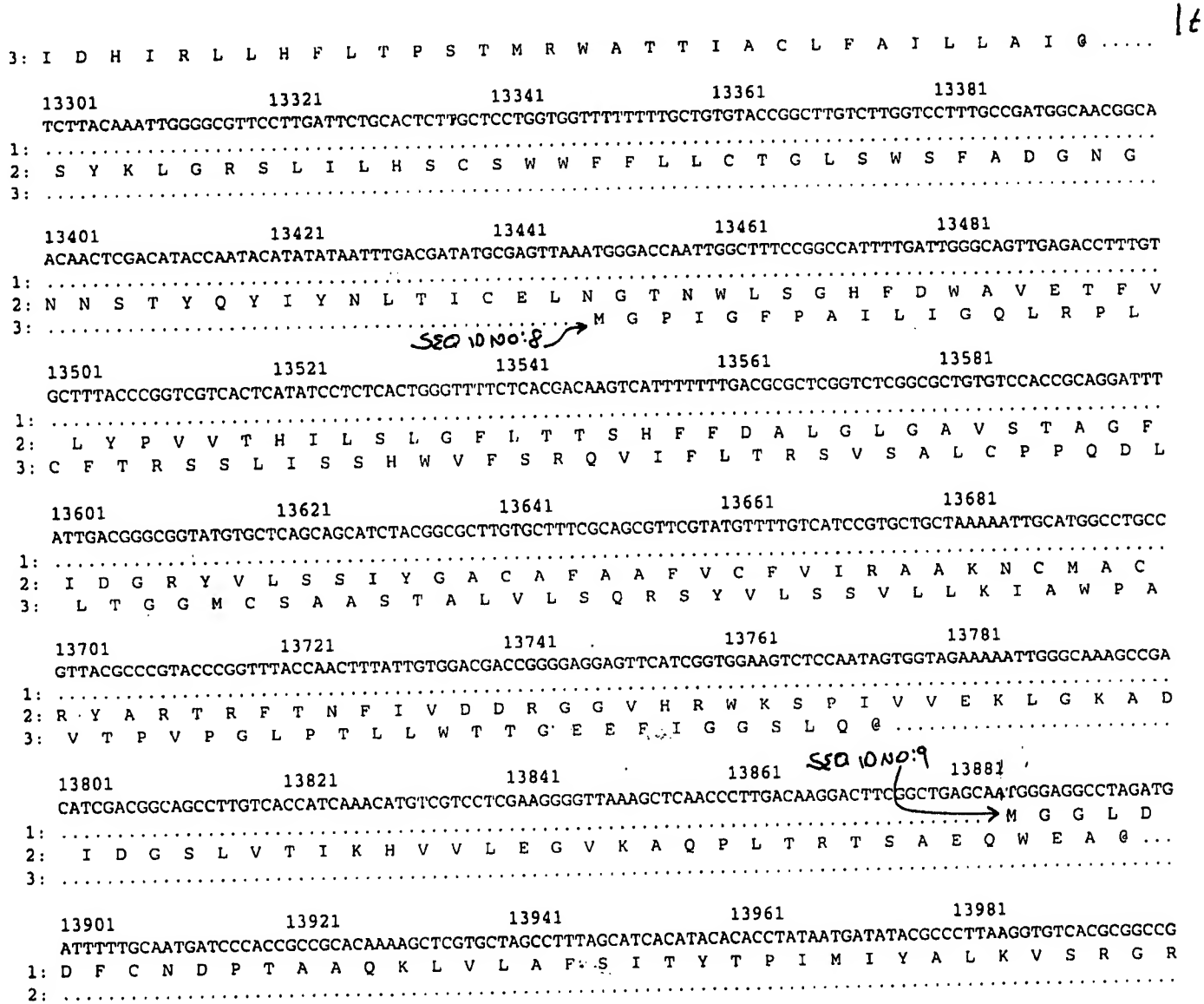
2: .....
3: .....
12701 12721 12741 12761 12781
ATTACCACCACCAAATAGACGGGGGTAATTGGTTTCATTTGGAATGGCTGCGGCCATTCTTTTCCTCCTGGCTGGTGCTCAACATATCATGGTTTCTGAG
1: Y Y H H Q I D G G N W F H L E W L R P F F S S W L V L N I S W F L R
2: .....
3: ..... M A A A I L F L L A G A Q H I M V S E
SEQ ID NO:6
12801 12821 12841 12861 12881
GCGTTCGCCTGTAAGCCCTGTTTCTCGACGCATCTATCAGATATTAAGACCAACACGACCGCAGCTGCCGGTTTCATGGTCCTTCAGGACATCAATTGTT
1: R S P V S P V S R R I Y Q I L R P T R P Q L P V S W S F R T S I V
2: .....
3: A F A C K P C F S T H L S D I K T N T T A A A G F M V L Q D I N C
12901 12921 12941 12961 12981
TCCGACCTCATGAGGTCTCAGCAACGCAAAGGGAAATTCCCTTCAGGAAGTCGTCCCAATGCCGTGAAGCCGTCGGCACTCCCCAATATATCACGATAAC
1: S D L M R S Q Q R K G K F P S G S R P N A V K P S A L P N I S R @ .
2: .....
3: F R P H E V S A T Q R E I P F R K S S Q C R E A V G T P Q Y I T I T
13001 13021 13041 13061 13081
AGCTAACGTGACCGACGAATCATATTTGTACAACGCGGATTTGCTGATGCTTTCTGCGTGCCTTTTCTACGCTTCAGAAATGAGCGAGAAAGGCTTCAAA
1: .....
2: .....
3: A N V T D E S Y L Y N A D L L M L S A C L F Y A S E M S E K G F K
13101 13121 13141 13161 13181
GTCATCTTTGGGAATGTCTCTGGCGTTGTTTCTGCTTGTGTCAATTTCACGGATTATGTGGCCCATGTGACCCAACATACCCAGCAGCATCATCTGGTGA
1: .....
2: .....
3: V I F G N V S G V V S A C V N F T D Y V A H V T Q H T Q Q H H L V
13201 13221 13241 13261 13281
TTGATCACATTCGGCTGCTGCATTTCCTGACACCATCTACAATGAGGTGGGCTACAACCATTGCCTGTTTGTTCGCCATTCTCTTGGCGATATGAGATGT
1: .....
2: ..... M R C
SEQ ID NO:7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1: .....
2: S Y K L G R S L I L H S C S W W F F L L C T G L S W S F A D G N G
3: .....
13401 13421 13441 13461 13481
ACAACTCGACATACCAATACATATATAATTTGACGATATGCGAGTTAAATGGGACCAATTGGCTTTCCGGCCATTTTGATTGGGCAGTTGAGACCTTTGT
1: .....
2: N N S T Y Q Y I Y N L T I C E L N G T N W L S G H F D W A V E T F V
3: ..... M G P I G F P A I L I G Q L R P L
SEQ ID NO:8
13501 13521 13541 13561 13581
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1: .....
2: L Y P V V T H I L S L G F L T T S H F F D A L G L G A V S T A G F
3: C F T R S S L I S S H W V F S R Q V I F L T R S V S A L C P P Q D L
13601 13621 13641 13661 13681
ATTGACGGGCGGTATGTGCTCAGCAGCATCTACGGCGCTTGTGCTTTCGCAGCGTTCGTATGTTTTGTCATCCGTGCTGCTAAAAATTGCATGGCCTGCC
1: .....
2: I D G R Y V L S S I Y G A C A F A A F V C F V I R A A K N C M A C
3: L T G G M C S A A S T A L V L S Q R S Y V L S S V L L K I A W P A
13701 13721 13741 13761 13781
GTTACGCCCGTACCCGGTTTACCAACTTTATTGTGGACGACCGGGGAGGAGTTCATCGGTGGAAGTCTCCAATAGTGGTAGAAAAATTGGGCAAAGCCGA
1: .....
2: R Y A R T R F T N F I V D D R G G V H R W K S P I V V E K L G K A D
3: V T P V P G L P T L L W T T G E E F I G G S L Q @ .....
SEQ ID NO:9
13801 13821 13841 13861 13881
CATCGACGGCAGCCTTGTCACCATCAAACATGTCGTCCTCGAAGGGGTTAAAGCTCAACCCTTGACAAGGACTTCGGCTGAGCAATGGGAGGCCTAGATG
M G G L D
1: .....
2: I D G S L V T I K H V V L E G V K A Q P L T R T S A E Q W E A @ ...
3: .....
13901 13921 13941 13961 13981
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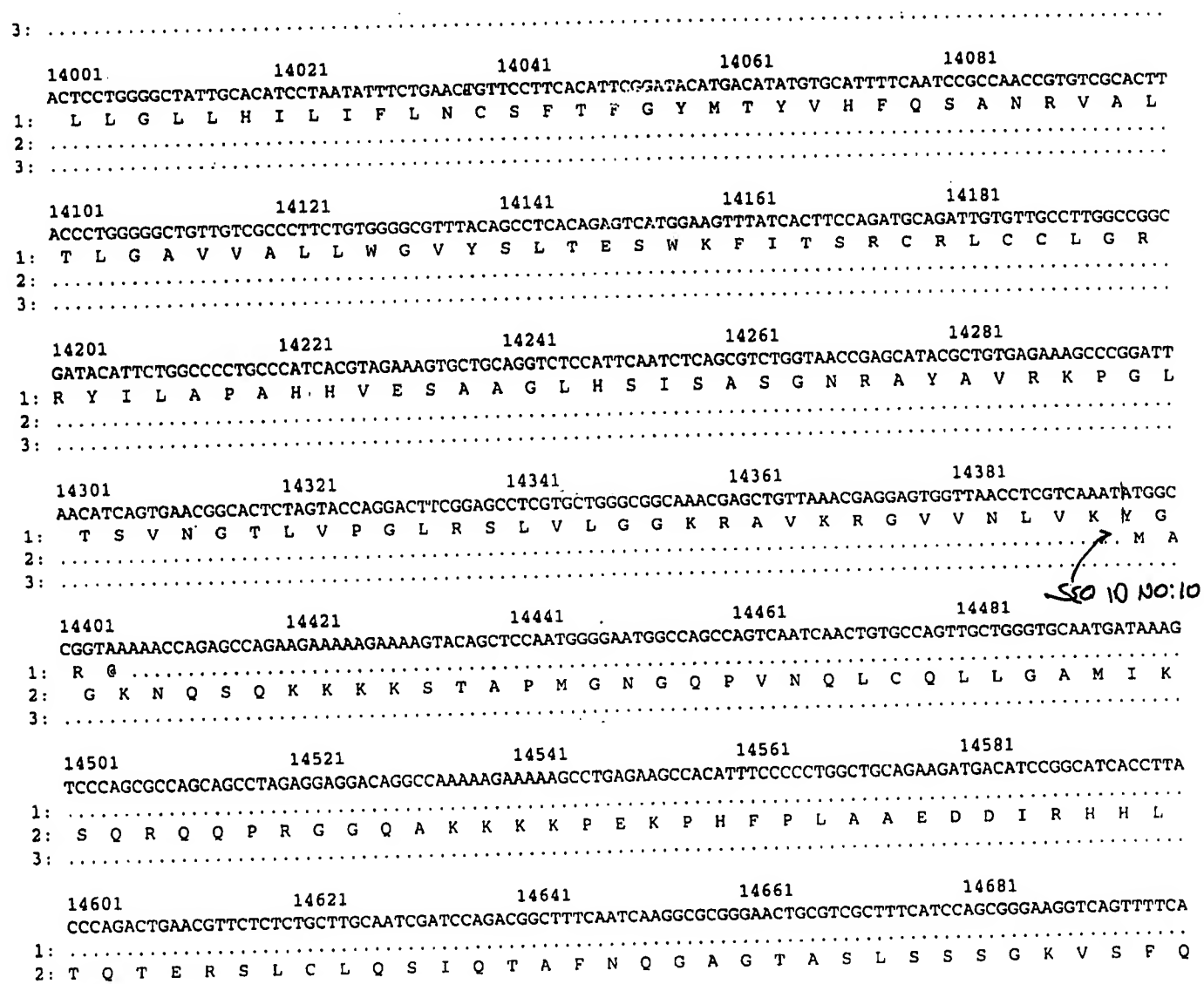
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2:
3:
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2:
3:
14201 14221 14241 14261 14281
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2:
3:
14301 14321 14341 14361 14381
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2:
3: M A
14401 14421 14441 14461 14481
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1: R @
2: G K N Q S Q K K K K S T A P M G N G Q P V N Q L C Q L L G A M I K
3:
14501 14521 14541 14561 14581
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1:
2: S Q R Q Q P R G G Q A K K K K P E K P H F P L A A E D D I R H H L
3:
14601 14621 14641 14661 14681
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1:
2: T Q T E R S L C L Q S I Q T A F N Q G A G T A S L S S S G K V S F Q

SEQ ID NO:10

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3: ..................................................................................................

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1: ..................................................................................................
2:  V  E  F  M  L  P  V  G  H  T  V  R  L  I  R  V  T  S  T  S  A  S  Q  G  G  A  S  Θ  .....................
3: ..................................................................................................

      14801          14821          14841          14861          14881
   GGTCGCGATTGGCGTGTGACCTCTGAGTCACCTATTCAATTAGGGCGATCACACATGGGGGTCATACTTAATCAGGCAGGAACCATGTGACCGAAATT

1: ..................................................................................................
2: ..................................................................................................
3: ..................................................................................................
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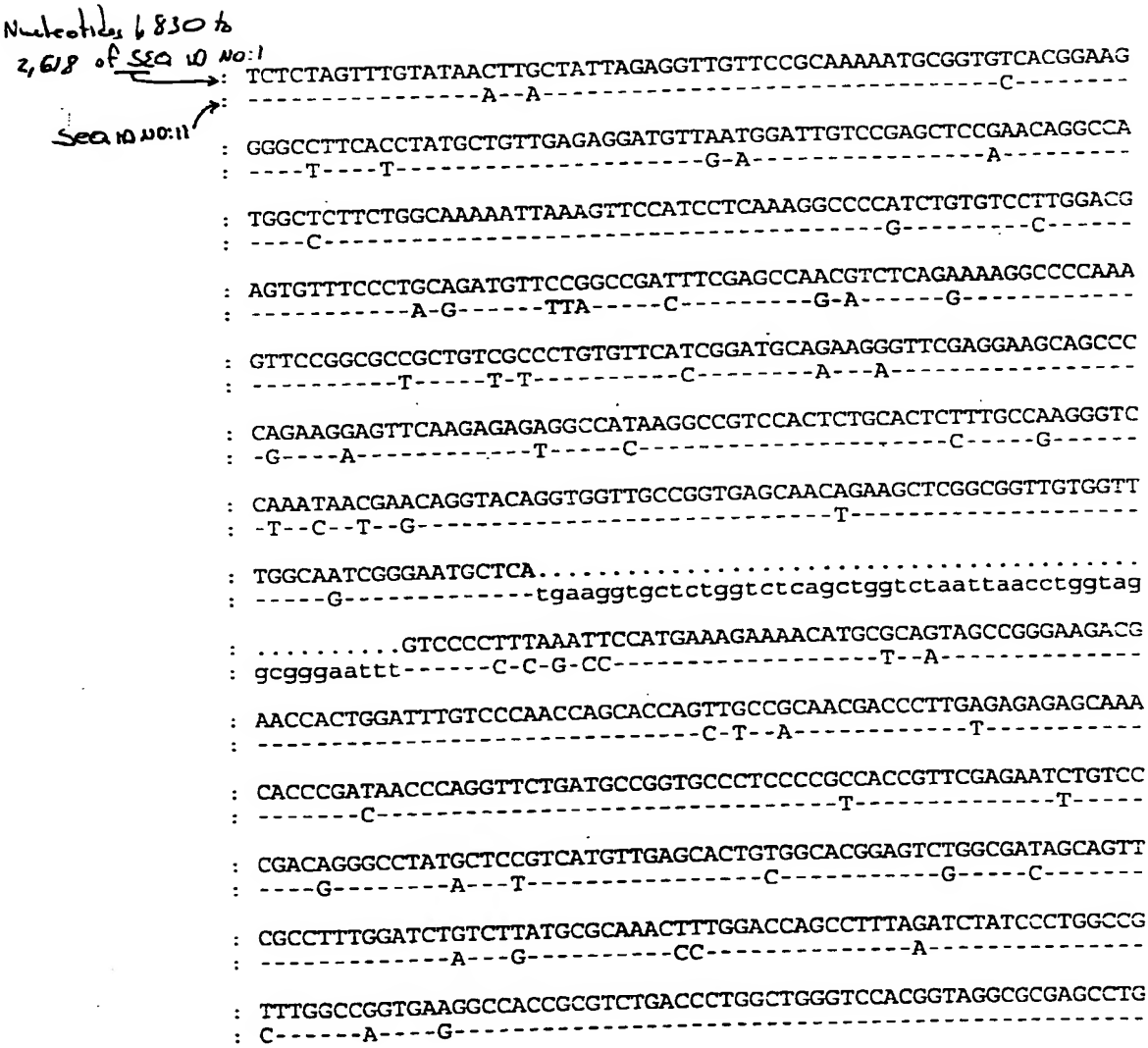
Fig. 2.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----C----------G---------C------
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----------T-----T-T----------C--------A---A----------
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-G----A----------T-----C----------C-----G------
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.....GTCCCCTTTAAATTCCATGAAAGAAAACATGCGCAGTAGCCGGGAAGACG
gcgggaattt------C-C-G-CC----------T--A----------
AACCACTGGATTTGTCCCAACCAGCACCAGTTGCCGCAACGACCCTTGAGAGAGAGCAAA
----------C-T--A----------T----------
CACCCGATAACCCAGGTTCTGATGCCGGTGCCCTCCCCGCCACCGTTCGAGAATCTGTCC
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CGACAGGGCCTATGCTCCGTCATGTTGAGCACTGTGGCACGGAGTCTGGCGATAGCAGTT
----G--------A---T----------C----------G-----C-------
CGCCTTTGGATCTGTCTTATGCGCAAACTTTGGACCAGCCTTTAGATCTATCCCTGGCCG
----------A---G----------CC----------A----------
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C------A----G----------

INTERNATIONAL SEARCH REPORT

Int ational Application No
PC f/US 01/04351

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N7/00 C07K14/08 A61K39/42 A61K39/12 A61P31/14
C12N15/40 G01N33/569 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, MEDLINE, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 21375 A (STICHTING CENTR DIERGENEESKUND) 10 December 1992 (1992-12-10) cited in the application the whole document	
A	WO 96 40932 A (PAUL PREM S ;HALBUR PATRICK (US); MENG XIANG JIN (US); MOROZOV IGO) 19 December 1996 (1996-12-19)	
X	claim 18	42
A	WO 96 04010 A (UNIV MINNESOTA) 15 February 1996 (1996-02-15) the whole document	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 2001	20/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Niemann, F

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int' ational Application No
PCT/US 01/04351

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